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(54) Title: LECTIN COMPOSITION AND METHOD FOR DIAGNOSIS OF CANCER

(57) Abstract

A lectin such as peanut lectin, lectin extract of orange peel, Maclura pomifera lectin, Dolichos Biflorus agglutinin and Soybean agglutinin conjugated with a therapeutic agent or a radiolabel can be utilized to detect cancer cells or malignant tumors by scintigraphy can be used to therapeutically treat cancer cells or malignant tumors. A kit is also provided to produce the therapeutic or diagnostic compositions.

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LECTIN COMPOSITION AND METHOD FOR DIAGNOSIS OF CANCER

Background of the Invention

Nuclear medicine plays an important role for the clinical oncologist, providing a non-invasive diagnostic procedure capable of rapidly screening the entire body for cancer, if appropriate radiopharmaceuticals are available. Tumor scanning has become an established method for assessing the initial extent of spread (stage) of primary and metastatic lesions and providing a means of evaluating the efficacy of a therapeutic procedure. It is desirable for the radiopharmaceutical to exhibit a selective uptake in the tumor in order to achieve a tumor:background ratio, which permits detection of the neoplastic lesions. Typically, when the tumor:background ratio is 2:1, tumor lesions will only be detected if the diameter is larger than about 2 centimeters. With improved concentration ratios, smaller lesions may be detected successfully and thus allow for earlier treatment of the cancer.

The growth of tumor masses may alter tissue physiology or displace normal tissue, leading to abnormal scans with commonly used radiopharmaceuticals. These "nonspecific limited-use" agents are able to detect physiological changes, such as: space occupying lesions in ^{99m}Tc sulfur colloid liver images, increased permeability of the blood brain barrier in ^{99m}Tc (pertechnetate and its chelates) brain scans and increased bone mineral metabolism in ^{99m}Tc-phosphate bone scans. All of these methods are unfortunately nonspecific and the scan abnormalities may also be due to nonmalignant processes. In addition, only one organ or tissue system is imaged, so that different radiopharmaceuticals are required for the evaluation of various organs.

Some tumor imaging agents attempt to exploit a qualitative or quantitative difference of tumor tissue which distinguishes it from normal tissue. Labelled metabolites can be tailored to a particular tumor. Thus, iodocholesterol labelled with ^{131}I is known to accumulate in cortisol-secreting adrenal tumors and ^{75}Se-selenomethionine localizes in protein synthesizing hepatomas and parathyroid adenomas. Melanin-avid chloroquine analogues successfully localize in melanomas and thyroid lesions can be imaged with radioiodine or ^{99m}Tc pertechnetate. Most of these agents are not useful for routine tumor diagnosis, because they fail to interact with a broad spectrum of tumor types.

Certain antibiotics have been known to affect the growth of tumors and, therefore, attempts have been made to label them, for use as tumor localizing agents. Tetracycline, labelled with either ^{99m}Tc or ^{131}I, is concentrated somewhat in tumors but its uptake in nonmalignant tissue has precluded any extensive use. Bleomycin, an anti-tumor antibiotic, has been labelled with a wide variety of radionuclides including ^{57}Co, ^{111}In, ^{123}I and ^{99m}Tc. Radiolabelled bleomycin localizes in a broad spectrum of neoplasmas and demonstrates a rapid blood clearance (dependent upon the in vivo stability of the label). However, the bleomycin complexes are not tumor-specific and also localize in nonmalignant lesions, such as inflammatory processes.

Excellent tumor visualization has been achieved with ^{67}Ga for some tumor types, particularly bronchogenic carcinoma, lymphoma and Hodgkin's disease, hepatoma and melanoma. Unfortunately, other common tumors (breast, gynecological, gastrointestinal and genitourinary tract tumors) are often imaged with gallium with inconsistent success. The biodistribution of radiogallium is relatively complex, with the liver, bone marrow, stomach, intestine, nasopharynx and salivary glands concentrating an appreciable fraction. Localization of gallium in the

gastrointestinal tract often obscures detection of neoplastic lesions in the abdominal cavity. Other disadvantages include the expense of radiogallium and the sub-optimal physical decay characteristics, which reduce the image quality. The major drawback, in the routine use of ^{67}Ga-citrate, is its lack of specificity. It accumulates in a wide variety of benign tissues and inflammatory lesions, thus complicating the diagnosis.

The major reason for the nonspecificity of the aforementioned tumor localizing agents stems largely from their mechanism of localization. The altered regional physiology of tumors, including increased vascular permeability and interstitial space and decreased lymphatic drainage, also occurs in nonmalignant disease states. Therefore, localization in benign lesions, as well as in tumors, is to be expected for those radiopharmaceuticals in which localization is effected primarily as a result of altered microvascularity or nonspecific trapping.

Neoplastic alteration of a cell is often associated with changes in the cell surface, which result in the expression of new protein or glycoprotein antigens. Antibodies, which bind specifically to these neoantigens, can be radioactively labelled to provide agents for tumor imaging with the important property of specificity. Recently, a new hybrid discipline of nuclear medicine and immunology has developed, specializing in the radioimmunodetection of cancer. By virtue of the selectivity of such antibodies, they may also be potentially used as carriers of cytotoxic or chemotherapeutic drugs or therapeutic doses of radioactivity.

Many immunopharmacological factors are involved in the distribution of a radioantibody. Each component of the system, antigen, antibody and radionuclide, has a very important contribution to the final success of tumor localization. A summary of important factors in radioimmunodetection have been discussed by various investigators and are summarized in Table I.

TABLE I

Major Considerations for Successful Radioimmunodetection of Tumors

Component	Required Characteristic
antigen and tumor characteristics	1) accessibility of antigenic site - antigen must be present on cell surface, rather than intracellularly - antigenic-site not blocked by host antibodies 2) tumor specificity of antigen marker - antigen present only on primary and metastatic tumors and not on benign or normal tissues 3) long retention of antigen on cell surface - antibody retention time at tumor sufficient for imaging - antigens shed from tumor may interact with antibodies in circulation 4) localization extent possible in tumor - perfusion, permeability, extravascular space and lymphatic drainage of particular tumors sufficient to allow for antibody localization
antibody (or lectin)	1) consistent specificity; reactivity only with tumor antigen of interest - pure antigen required to produce antibodies of specific reactivities - production, selection and purification methods capable of producing specific antibodies

TABLE I (cont)

Component	Required Characteristic
antibody (or lectin)	2) nonspecific localization is controlled or accounted for - simultaneous injection of nonspecific antibody labelled with a different isotope 3) high tumor/background ratio for good image contrast - fast blood clearance - low binding to backgroun tissue 4) nontoxic and nonallergenic - repeated injections of foreign proteins could induce anaphalaxis
labelling or imaging technique	1) choice of radionuclide - chemical and physical properties - appropriate effective half-life of radiopharmaceutical - radionuclide amenable to scintographic techniques - radiation dose within acceptable limits 2) labelling technique - required levels of specific activity are attained - biological activity retained following radiolabelling procedure - radiochemical stability 3) special imaging techniques - system capable of subtraction methods for nonspecific localization and blood pool activity (e.g., $^{99m}TcO_4^-$ and ^{99m}Tc-human serum albumin)

Initial studies into the use of antibodies as tumor-localizing agents indicated that these agents suffered from various limitations. ^{131}I-rabbit antibody to human fibrinogen showed promising results but lacked tumor specificity. Antibodies produced against simgle cell homogenates, such as renal cell carcinoma, requires the production of antibodies to individual tumors and tumor types. This must be followed by extensive work-up to remove cross-reacting antibodies. The production of different antibody preparations for each individual patient is not practical, from the viewpoint of time-cost effectiveness and patient delay.

The carcinoembryonic antigen (CEA) is a well-studied oncofetal antigen. It is a component of embryonic and fetal gut and is also found in human gastrointestinal (GI) carcinomas. CEA has been subsequently found in a variety of normal as well as malignant tissues. Nevertheless, quantitatively increased plasma CEA levels have been found to be clinically significant in most GI cancers. ^{125}I- and ^{131}I-labelled goat anti-CEA also have been used successfully to localize xenografts of human colonic carcinoma in hamsters. A delay of 4 to 10 days post-injection was required for optimum tumor localization, due to a slow clearance of background radioactivity of the blood and non-tumor tissues.

Successful clinical tumor imaging with anti-CEA was achieved only when blood pool background was subtracted by a computerized technique. Simultaneous injection of ^{99m}-Tc-HSA (human serum albumin) and ^{99m}Tc-pertechnetate permits computerized-subtraction of the blood pool and free radioiodide from the radiolabelled antibody distribution. Tumor:non-tumor ratios of radioactivity were only 1:4 prior to background subtraction in one study but the tumor images were enhanced about 2.5 times following computer-assisted processing of the images. The absolute requirement of this subtraction technique for successful tumor imaging has led some investigators to question the present suitability of anti-CEA for routine clinical use.

Radiolocalization studies have also been reported which use antibodies to human chorionic gonadotropin (HCG) and alpha-fetoprotein (AFP). As with anti-CEA, a slow blood clearance in these investigations did not permit tumor-specific localization significantly above background tissue. Therefore, computer-assisted background subtraction and special calculations were required to demonstrate tumor selectivity of the antibodies.

The principal difficulties with the use of radioactively-labelled antibodies have been (1) the preparation of ultraspecific antibodies to tumors and (2) suppressing the background radioactivity caused by unbound antibody or antibody-antigen complexes in the circulation. Upon injection of an antigen macromolecule into an animal, many cross-reactive antibodies are synthesized, due to the availability of numerous antigenic sites on a macromolecule. The resulting antibody preparation will not be homogeneous and will be able to detect and react with many more antigens than the specific desired tumor marker. Attempts to rectify this problem have involved the isolation and purification of the specific antigenic determinant of the tumor marker and the production of monoclonal antibodies.

Kohler and Milstein developed a method in which hybridomas were used to produce homogeneous and reproducible monoclonal antibodies which are reactive to only one antigenic determinant. Antibody-secreting plasma cells, specific for the antigen, are hybridized or fused to myeloma cells which provide the ability to grow continuously in culture as hybridoma cells. Monoclonal antibodies have been developed towards various tumor markers and have been used in the radioimmunodetection of the metastases of hepatoma, colorectal cancer and choriocarcinoma. Unfortunately, the technique is relatively complicated, sophisticated and labor intensive.

Accordingly, it would be desirable to provide reagents which are more specific to tumor antigen markers than antibodies so that the tumor:non-tumor ratio of uptake of the reagent is a higher value than the uptake value of presently available reagents including radiolabelled antibodies. Such reagents would permit the diagnosis and therapy of malignant cells and tumors in vivo or in vitro in a manner more selective than presently available processes.

Summary of the Invention

In accordance with this invention, radiolabelled compositions are provided which comprise a lectin selected from the group consisting of peanut lectin (PNA), extract of orange peels, Maclura pomifera (MPA), Dolichos Biflorus agglutinin (DBA), Soybean agglutinin (SBA) or other lectins of plants, animals or fish origin or active subunits of these lectins bound to antigenic markers on malignant cells or tumors, which compositions are either conjugated with a therapeutic agent or with a radiolabel. These compositions are administered parenterally to humans and, in the case of radiolabelled compositions, the biodistribution of the labelled composition is monitored by scintigraphy in order to locate cancer cells or malignant tumors. In the case of compositions conjugated with a therapeutic agent, the conjugated composition interacts specifically with the malignant cells or tumor in order to specifically deliver the therapeutic agent to the cell to kill the cell or shrink the tumor. The present invention provides substantial advantages over the prior art since the compositions utilized are more specific toward cancer cells or malignant tumors than compositions including an antibody to a tumor-specific antigen. A kit is also provided which includes the PNA lectin, the MPA lectin or subunits thereof as well as reagents necessary to radiolabel the lectin or subunit thereof including the radiolabel.

Description of Specific Embodiments

The word lectin comes from the Latin legere meaning to pick out or choose. The term was initially applied to a group of carbohydrate-binding plant seed (glyco) proteins which could distinguish among human blood groups. Sugar-binding proteins, which interact with an agglutinate plant and animal cells, have now been identified in a diverse range of organisms, including: bacteria, molds, and algae, plants, sponges, fish, snails, eels, crabs and even mammals.

Many lectins currently employed are purified to homogeneity by affinity chromatography on columns containing carbohydrate supports. Studies of those lectins obtained in purified form, have revealed a diverse range of physical and chemical characteristics. There is no structural feature common to all lectins, except that they are proteins. All lectins consist of subunits, although the number of subunits per molecule varies. Some lectins require metal ions such as Ca^{+2} or Mn^{+2} for biological action. Molecular weights of lectins have ranged from 36,000 to 335,000. Sometimes the lectins occur as a group of closely related proteins, called isolectins, which have very similar chemical and biological properties but differ in electrophoretic mobility. Many of the lectins are glycoproteins, although several lectin proteins, such as peanut lectin, concanavalin A and wheat germ, lack covalently linked sugars. did not work

Lectins are similar to antibodies in many respects. The combining site of the lectin interacts specifically with the carbohydrate-bearing structure, to induce agglutination or precipitation. This is similar to antibody-antigen reactions because it is very specific and also reversible. In addition, the interaction can be specifically inhibited by low molecular weight haptens which block the combining site.

The binding site of the lectin is usually smaller than that of an antibody. Simple monosaccharides can often inhibit lectin interactions, but the smallest fragment which will inhibit precipitation by antibodies is a disaccharide.

Other major differences exist between antibodies and lectins. Antibodies are glycoproteins secreted by lymphocytes, as an elicited response to a foreign stimulus. In contrast, the organisms in which lectins originate, are often not able to produce an immune response and the lectins are usually present as constituent proteins. While the specificity of lectins is restricted to simple and complex carbohydrates, antibodies can be formed to react specifically with other classes or organic compounds, including amino acids, proteins and nucleic acids. The antibodies that are formed are structurally similar. This is in contrast to the structural diversity observed for lectins isolated from various sources.

In spite of these differences, lectins have often been used as models of carbohydrate-specific antibodies and have been used to replace them in techniques such as blood grouping. Lectins are readily available and are easily purified in gram quantities. In addition, the combining site of purified lectins, unlike that of most immune antibodies, is small and homogeneous.

The overall carbohydrate specificity of a lectin is usually defined by the monosaccharides of oligosaccharides that have the strongest inhibitory effect on the lectin-induced agglutination or precipitation reactions.

Lectins usually have stringent structural requirements for optimal binding. Often the C-3 and C-4 hydroxyl groups of sugars appear to play an important role in lectin binding. The generality that D-galactose binding lectins do not usually interact with D-glucose or D-mannose specific lectins and vice versa demonstrates the critical involvement of the C-3 hydroxyl moiety.

Although the binding site of most lectins accomodates a single glycosyl residue, some lectins have extended saccharide binding sites. The peanut lectin (Arachis hypogoea), has a binding site complimentary to a disaccharide and the affinity

of wheat germ agglutinin (Triticum vulgaris) for β(1→4)-linked D-GluNAc increases for the trisaccharide series of the same sugar residue. The orange peel lectin, Maclura pomifera (MPA) has a binding site characterized by α(1→3) GalNAc and Gal. Dolichos Biflorus agglutinin binds to α-N-Acetyl-D-Galactosamine residue, and the Soybean agglutinin binds to the α-N-Acetyl-D-Galactosamine as well as to the Galactose moieties.

The binding of lectins to complex oligosaccharides, glycoproteins and cells is a more complex phenomenon than that seen with simple sugars. Multivalent and secondary nonspecific interactions are superimposed on the primary, carbohydrate-specific binding. Therefore, the association constants for lectin-glycoprotein binding is usually several orders of magnitude higher than those found for monosaccharides.

Although most studies involving lectins have been in vitro, lectins have been used in vivo as carriers for the delivery of chemotherapeutic agents to tumors. The lectin which has often been utilized as a carrier of antitumor drugs, has been concanavalin A. Concanavalin A (con A) has its own antitumor activity. It selectively agglutinates and kills some tumor cells in vitro and can increase the life span of tumor-bearing mice. Injection of a con A chemotherapeutic drug complex was found to be more effective in prolonging survival of tumor bearing mice than injection of either the lectin or antitumor drug alone. Since con A is a powerful mitogen and may induce haemorrhagic Arthus-like reaction, hydrocortisone was sometimes given concurrently with the con A complexes.

The peanut (Arachis hypogoea) lectin is readily available protein, which binds preferentially to oligosaccharides containing the terminal sequence β-D-galactosyl-(1→3)-α-N-acetyl-D-galactosamine (βDGal(1→3)GalNAc). This disaccharide is reported to be the immunodeterminant group of the Thomsen-Friedenreich antigen (T antigen). The T antigen is present in a number of membrane and soluble glycoproteins, but it is nor-

mally masked by N-acetyl-neuraminic (sialic) acid residues. Therefore, the peanut lectin will only bind to the glycoproteins following exposure of the T-antigen by neuraminidase treatment.

The affinity of the peanut lectin (PNA) for the T antigen, allows this protein to agglutinate neuraminidase-treated red blood cells. It was, therefore, designated an "anti-T agglutinin" since it gave the same immunological reaction as the anti-T antibody of mammalian sera. A special importance and clinical relevance became associated with the T antigen, following its discovery in the cell membranes of human carcinomata. The tumor-associated T specificity has been found in the unmasked, reactive form in adenocarcinoma of the breast, gastrointestinal and respiratory tract. The expression of the T antigen on malignant tissue, and not on corresponding benign or normal tissue, may represent incomplete synthesis or accelerated degradation.

Affinity-purified peanut lectin (PNA) has been determined to be homogeneous. The intact peanut lectin protein exhibits a molecular weight of 110,000 ± 10,000 as determined by gel filtration and sedimentation velocity. This value was in close agreement with a value of 106,500 determined by sedimentation equilibrium centrifugation. Analysis of the lectin by sedimentation equilibrium and also gel filtration under a wide range of pH and concentration ranges, showed a lower molecular weight value of 98,000 ± 3,000. Possible reasons for this discrepancy could be (1) method of extraction leading to slight aggregation, (2) higher protein concentrations used (2.5 - 10 mg/ml) may result in a lower $S^{\circ}_{20}{}^{\omega^{69}}$, which results in a higher estimate of molecular weight and (3) isolectin variation.

The peanut lectin has recently been crystallized, for x-ray studies, into an asymmetric orthorhombic crystal with 57% of the volume being solvent-occupied. It is a relatively acidic (pI = 5.95) hydrophilic, and compactly folded globular

protein. The lectin is believed to be composed of four identical monomers of 24,500-27,000 dalton molecular weight, which are non-covalently linked. There are 4 binding sites per molecule (revealed by equilibrium dialysis) and metal analysis by atomic absorption analysis indicates that each PNA subunit contains one Ca^{+2} and $Mg^{+}2/Zn^{+2}$ atom (0.78 mole Mg^{+2} per subunit and 0.11 mole Zn^{+2}/subunit).

The quaternary structure of PNA is pH dependent. As the pH is gradually decreased from 4.75 to 3.0, the molecule reversibly dissociates from a tetramer into a dimer. The 48,000 dalton species which exists at pH 3 lacks sugar binding ability. Denaturing conditions or detergents (sodium dodecylsulfate) dissociate the intact molecule into its 4 subunits, which have identical molecular weights and identical sequencing for the last five NG_2-terminal amino acids.

The first 40 amino acids of peanut lectin N-terminus have been sequenced. It has been found that there is considerable homology between the first 25 and the first 40 residues of PNA and several other lectins isolated from legumes (e.g., soybean, lentil and pea).

Isolation of lecitns generally begins with a saline or buffer extraction of the finely-ground seed meal. The anti-T activity was initially discovered in crude saline extracts such as these.

As much as 40 per cent of the net weight of peanuts is composed of lipid components, some of which may be contained in the crude saline extracts. Therefore, a pre-extraction step with organic solvents (such as ether or acetone) is often employed to remove lipidic or other interfering substances. The proteins, from the defatted preparation, are then usually salted out with neutral salts such as 40-75% ammonium sulfate. The precipitate is then collected by centrifugation before being redissolved for dialysis and/or ultracentrifugation. The clear supernatant will contain a number of proteins besides the

lectin, such as the large molecule weight peanut globulins, known as arachin (MW~380,000) and conarachin. The lectin can be isolated from this protein mixture by conventional protein-purification techniques, affinity chromatography or a combination thereof.

Virtually most of the other contemporary lectin-purification schemes employ affinity chromatography techniques, which exploit the specific sugar-binding capacity of the lectin. A carbohydrate ligand with which the lectin interacts is insolubilized in some manner, so as to allow for specific adsorption of the lectin. The lectin can then be displaced by elution with a sugar which is competitive for the same binding site. The biospecific adsorbents of lectins may be divided into 3 major types: (1) native or modified polysaccharides, (2) matrix-bound glycoproteins or glycopeptides and (3) matrix-bound mono- and disaccharides. They all have in common terminally exposed galactose or lactose residues to which the peanut lectin can be specifically and reversibly bound. The immunodeterminant disaccharide (β-D-Gal (1→3)-α-D-GalNAc) of the T antigen has recently been synthesized by Chembiomed Ltd of Edmonton, Alberta, Canada into an immunoadsorbent form. Any conventional method for separation and purification of PNA, MPA, SBA and DBA can be utilized to produce the lectins employed in this invention.

This invention is suitable either for <u>in vitro</u> or <u>in vivo</u> testing.

Among the radioisotopes used, gamma-emitters, positron-emitters, x-ray emitters and fluorescence-emitters are suitable for localization and/or therapy, while beta-emitters and alpha-emitters may also be used for therapy. Suitable radioisotopes for labelling antibodies include Iodine-131, Iodine-123, Iodine-126, Iodine-133, Bromine-77, Indium-111, Indium-113m Gallium-67, Gallium-68, Ruthenium-95, Ruthenium-97, Ruthenium-103, Ruthenium-105, Mercury-107, Mercury-203, Rhenium-99m, Rhenium-105,

Rhenium-101, Tellurium-121m, Tellurium-122m, Tellurium-125m, Thulium-165, Thulium-167, Thulium-168, Technetium-99m, Fluorine-18, Astatine-211, Copper-67 and Copper-64. The halogens can be used more or less interchangeably as labels since halogen-labelled antibody fragments and/or normal immunoglobulin fragments would have substantially the same kinetics and distribution and similar metabolism.

A preferred radiolabel is either radioactive iodine, e.g., I-131, I-123, I-125 or technetium-99m. A preferred labelling technique utilizing radioiodine involves labelling with either Iodine-131 (I-131) or Iodine-123 (I-123) using an oxidative procedure wherein a mixture of radioactive potassium or sodium iodide and the lectin is treated with chloramine-T. This results in direct substitution of iodine atoms for hydrogen atoms on the lectin molecule, depending on the proportions of reagents and the reaction conditions. Alternatively, lactoperoxidase iodination may be used. The radioiodinated lectin may also be prepared through the addition of labelled halide to a tube which was previously coated with Iodogen™ and which contains a solution of the lectin. After the appropriate incubation period, the solution mixture is removed and the unreacted free iodide is separated from the labelled lectin.

The technetium-99m labelled lectin or its derivatives can be prepared by acidic, basic or neutral (ligand-exchange) radiolabelling techniques. In one particular and preferred aspect of this invention, the labelled lectin or its derivatives is obtained via a ligand exchange process. In this process, a solution of Technetium (IV) is prepared by mixing a solution of Technetium such as in the form of a pertechnetate (TcO_4^-) and saline with a stannous reducing solution, e.g., stannous fluoride-acetate having a pH between about 3 and 5.5. In this procedure, the stannous ions reduce Technetium (VII) to Technetium (IV). The reduced Technetium-99m first is chelated onto the top of a column of Sephadex G-25 (dextran cross-

linked with carboxy functionality) by passing the aqueous solution of Technetium-99m through the column. The solution has a pH between about 5.5 and 7.0. The column then is wahsed with saline to essentially remove free Pertechnetate (TcO_4^-) or unusual species of Technetium thereby leaving the Technetium-99m chelated or adsorbed or otherwise bound to the column. A physiologic solution of the lectin or its derivatives is then prepared with appropriate buffer so that the resultant solution has a pH between about 6 and 9, preferably between about 7 and 8. When operating within this pH range, denaturation of lectin is eliminated or minimized. The lectin or its derivatives is then added in a minimum volume to the top of the column where the Technetium-99m/Stannous complex is bound and where it is allowed to stand until the Technetium-99m is bound to the protein having stronger bonding sites than the column material. This usually occurs within about 30 minutes. The column then is washed to remove the labelled lectin or its derivatives. Washing can be effected with a known volume of human serum albumin diluted with 50/50 ACD or the like followed by a known volume of saline. In this manner, the volume or washing saline solution containing the labelled protein can be determined and the labelled protein can be collected. Impurities in the lectin will remain on the column or will be eluted at a rate different from that of the labelled, immunologically intact lectin or lectin derivative.

A second preferred method for forming Technetium-99m labelled lectin or its derivatives comprises direct labelling of the protein. In this method, a buffered solution is admixed with an acidic solution of $SnCl_2$ which is a reducing agent for pertechnetate. The buffered solution can comprise sodium and/or potassium phthalate, tartrate, gentisate, acetate, borate or mixtures thereof having a pH of between 4.5 and 8.0, preferably about 5.5. Tartrate is utilized to maintain the appropriate concnetration of stannous ion in solution to effect the

desired solution pH. The $SnCl_2$ preferably is added to the buffer as a solution with concentrated HCl. Thereafter, the solution is neutralized such as with sodium hydroxide to attain a pH of between about 4.5 and 8.0, preferably about 5.5. The lectin or its derivatives then is added to the neutralized solution in an amount to attain a concentration of protein up to just less than that which would begin to precipitate the protein in the buffer being used. In order to attain the desired degree of protein labelling, the resultant stannous ion, buffer, protein solution is allowed to incubate. For example, at room temperature, the incubation time should be at least about 15 hours, preferably at least about 20 hours. If desired, this solution can be heated moderately to reduce the incubation time. The solution then can be either freeze-dried and subsequently reconstituted for admixture with pertechnetate or can be admixed directly with pertechnetate solution to obtain the labelled protein. If desired, the resultant radiolabelled protein may be further purified to separate the labelled protein from free technetium such as by chromatography in a Sephadex column. However, this last step is unnecessary.

The present invention also provides a kit with which a user can prepare the technetium-labelled composition of this invention and administer it to a patient relatively quickly after preparation. The kit includes the lectin or its derivatives either in lyophilized form, frozen or liquid of suitable ionic strength and pH, and either containing or not containing a reducing agent. If without the reducing agent, the lectin can be admixed with a reducing solution or solid provided within the kit and in a separate container. Representative, suitable reducing agents are $SnCl_2$ or SnF_2 to be dissolved or already dissolved in an appropriate solution, such as sodium acetate/acetic acid, acidified deionized or distilled water, or the like, such that a reducing pH of about 3 to 5.5 is obtained when combined with Technetium-99m as Sodium pertechnetate.

Therefore, Technetium-99m as pertechnetate is either reduced in the presence of reducing agent prior to addition of the lectin or is reduced when added to the lectin containing reducing agent. The solution of labelled lectin is then suitable for administration to a patient.

In an alternative embodiment of this invention, the kit can include a container for a column of material which entraps or otherwise binds Technetium-99m such as Sephadex, Sepharose or cellulose. The column of this material also can contain the reducing agent for technetium or the reducing agent can be added thereto when it is desired to reduce the technetium.

In forming the technetium products of this invention, a solution of the technetium-99m as the pertechnetate is poured onto the column in order to bind the technetium thereon. A physiologically acceptable aqueous solution of the lectin or its derivatives then is poured onto the column in order to bind the technetium to the lectin. The labelled protein then is eluted from the column with saline or an otherwise appropriate buffer and is collected from the bottom of the column in a form suitable for intravenous administration to a patient. In an alternative embodiment, the eluted labelled protein is passed through a bed of anion exchange resin in order to remove free pertechnetate from the labelled protein, thereby to form a pure technetium-labelled lectin thereof, substantially free of radiochemical contamination. If desired, these anion exchange resins need not be part of the columns utilized for labelling, but can comprise a separate bed through which the labelled protein is passed.

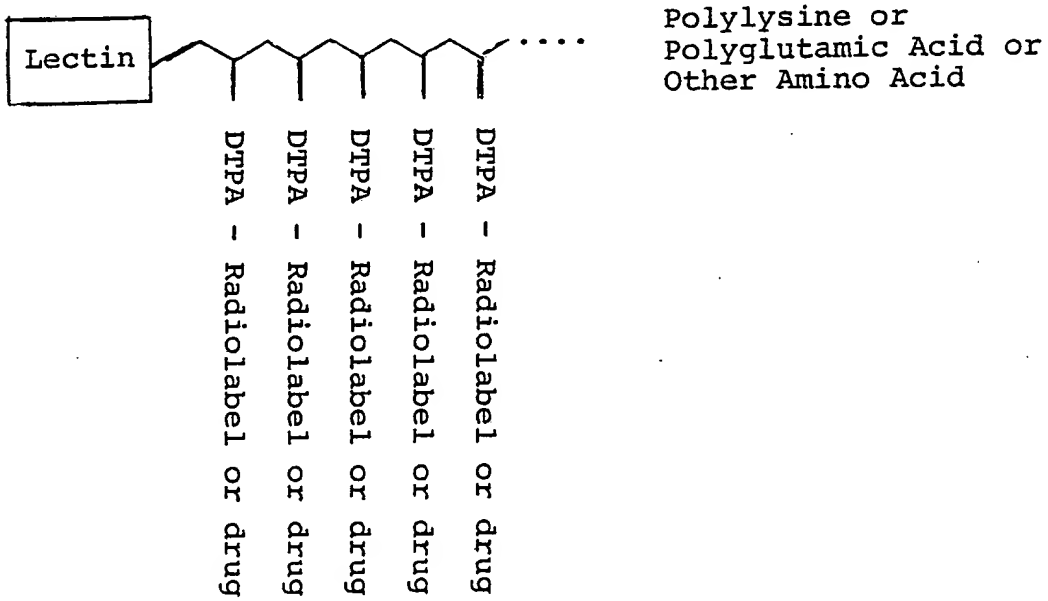
The labelled lectin or its derivatives is administered by intravenous injection in a pharmaceutically acceptable saline solution, sterile and pyrogen-free. Suitable dosages are usually between about 1 and 10 millicuries, preferably between about 2 and 10 millicuries of technetium-99m lectin for the

normal 70 kg patient. The patient then can be scanned by conventional scintigraphy within about 1 hour to about 6 hours after administration of the labelled protein. Tumors are located in those areas showing a high concentration of labelled lectin.

The following example illustrates the present invention and is not intended to limit the same.

The lectin derivatives may consist of conjugates in which either polylysine or polyglutamic acid or other amino acid residues of various molecular weights (500-50,000) are attached to the lectin molecule via a peptide bond. To these residues either radionuclides or other therapeutic agents can be attached to the lectin. Conceptually, the final molecule is as follows:

Lectin Derivative



Ethylenediaminetetraacetic acid (EDTA) can be utilized rather than DTPA as the linking agent.

Example I

This example illustrates the significantly increased specificity of peanut lectin for the Thomsen-Friedenreich (T) antigen which is a cell marker on many human adencarcinomata as compared to antibody to T antigen.

A. Preparation of Protein Solution

1. Anti-T IgG (rabbit)

The anti-T immunoglobulins used in these studies were provided by ChemBiomed, University of Alberta. An artificial T antigen, produced by coupling the synthetic T disaccharide to BSA (Ratcliffe et al, Carbohydr. Res., 95:35, 1981), for the immunization of rabbits. Following production of the high titre sera in the animals, anti-T IgG was affinity purified on an immunoadsorbent, also prepared from the synthetic T hapten.

The rabbit anti-T solution was provided at a concentration of 1.34 mg/ml in PBS (= phosphate buffered saline), (Gibco 0.01 M phosphate buffer in 0.15 M sodium chloride, pH 7.4).

2. Non-specific Rabbit IgG:intact and F(ab')2 Fragment

Chromatographically purified rabbit IgG was obtained in a lyophilized form with 0.1% azide preservative and was reconstituted with glass distilled water to a concentration of 25 mg/ml. The azide preservative was removed by 3 successive concentrations on an Amicon B-15 macrosolute concentrator (Amicon Corp., MA). Dilution with 0.4 M borate buffer, pH 7.4, was carried out between each concentration step. The protein and one wash solution were removed from the concentrator, added to 25 mg T immunoadsorbent (ChemBiomed, University of Alberta) and mixed by rotation gently for 2 hours at 4°C. The supernatant protein and a 0.4 M borate buffer wash of the T-sorb were aseptically passed through a 0.22 µM Millex-GS filter (Millipore Corp., Bedford, MA) into a sterile vial. The sterile IgG solution was aliquoted into sterile autoclaved ampoules and then sealed on a Cozzoli sealer before being stored at -15°C.

The rabbit $F(ab')_2$ IgG was purified in a similar manner as the intact IgG although in 1 to 6 mg lots. The Millipore filtered protein was aliquoted into sterile Falcon^R tubes (Can-Lab), stored at 4°C and used within 2-4 weeks.

3. Peanut Agglutinin

The peanut lectin was obtained from E.Y. Lab (San Mateo, CA) in salt- and sugar-free lyophilized form. The protein had been affinity purified (lactose-sepharose 4B column) to a pure single band on polyacrylamide disc gel electrophoresis.

The PNA was reconstituted with sterile PBS in a sterile Falcon tube to 1 mg/ml and passaged numerous times through a 26 gauge needle to break up the aggregates. The protein solution was then passed through a 0.22 µ Millipore filter before being placed in a previously autoclaved (121°C x 20 minutes) 1 cm UV cell (Thermal Syndicate Ltd., England). Optical density was measured at 280 nm in comparison to a PBS blank and the protein concentration was determined as described later. The solution was stored at 4°C, for no longer than two months.

B. Quantitative Protein Analysis

1. Rabbit IgG: Intact and $F(ab')_2$ Fragment

The protein concentration of the initially reconstituted antibody preparations was determined by a modification of the Folin-Phenol procedure (Lowry et al, J. Biol. Chem., 193:265, 1951 and Miller, Anal. Chem., 31:064, 1959). A standard curve of absorbance versus antibody concentration was prepared using nonspecific IgG immunoglobulin (Cappell Lab.).

The copper reagent was prepared freshly and consisted of: 1 part 1% W/V copper sulfate ($CuSo_4 \cdot 5H_2O$), 1 part 2% W/V aqueous sodium potassium tartrate and 20 parts 10% W/V sodium carbonate in 0.5 N sodium hydroxide. One ml of the copper reagent was added to one ml of the protein sample and following thorough mixing, it was incubated at room temperature for 10 minutes. Three ml of a 1:11 distilled water dilution of the

Folin-Phenol reagent (Fischer Sci. Co., Fair Lawn, NJ) was then added, thoroughly mixed and incubated at 50°C for 10 minutes. The absorbance of the solutions was read at 640 nm on a Unicam SP1800 Spectrophotometer (CanLab) using a blank prepared in the same manner, but substituting PBS for the protein solution. A standard curve of increasing protein concentrations, from 1 µg/ml to 200 µg/ml, against optical density was then used to determine the sample concentration.

Separate standard curves were prepared for both the intact and $F(ab')_2$ fragment of IgG proteins using the same respective immunoglobulins (but nonspecific IgG) as standards.

The protein concentration of the antibody solutions was also determined by ultraviolet absorption at 280 nm. The absorbance of the solutions, against a saline blank, was determined on a Unicam SP 1800 UV Spectrophotometer. The concentration of the immunoglobulin solutions was calculated by means of the absorbance coefficients of $E^{1\%}_{1\ cm} = 14.6$ for intact rabbit IgG and $E^{1\%}_{1\ cm} = 14.8$ for $F(ab')_2$ fragment IgG[233].

2. Peanut Lectin

Optical density, at 280 nm, was also used for determination of peanut lectin concentrations, using the method described for antibody preparations. An absorbence coefficient of $E^{15}_{1\ cm} = 9.6$[69] was used to convert absorbence readings to a concentration value.

C. Radiolabelling of Proteins

1. Quality Control of Radioiodine Solutions

The radionuclidic purity of $Na^{125}I$ solutions (Iodination grade, AECL) was confirmed using a 50 kBq aliquot in a NaI(Tl) well crystal and a multichannel analyzer (Canberra, Series 40 MCA) and comparing the gamma spectra obtained to that of a published standard (J. Radioanal. Chem., 65:341, 1981).

The radiochemical purity of the $Na^{125}I$ was confirmed using instant thin layer chromatography. Twenty kBq was spotted

on Gelman^R silica gel, instant thin layer 20 cm strips, air dried and developed in an 85% methanol in water solvent system. A chromatogram scanner was used to analyze the strips for distribution of radioactivity.

2. Radioiodination Procedures

a) Protected Anti-T IgG Immunoglobulin

Anti-T rabbit IgG immunoglobulin was prepared by ChemBiomed, University of Alberta. Anti-T IgG (180 µg) was placed in a 3 ml vial with 60 mg T-sorb (ChemBiomed) and 1 ml 0.5 M phosphate buffer, pH 7.2. This mixture was gently rotated for 2 hours at 4°C. The T-sorb was washed (3x) with 1 ml aliquots of 0.5 M phosphate buffer. Following removal of the final wash, the T-sorb-bound anti-T was labelled, in 1 ml of PO_4^- buffer, by a modified iodine monochloride method (Samols et al, Nature, 190:1211, 1961). Three to six µl of $Na^{125}I$ (iodination grade, AECL) was added and the mixture was gently agitated for 30 seconds before the addition of 25 µl of a 1:50 dilution of stock iodine monochloride solution (2.4×10^{-3} M). The iodination of the T-sorb-bound antibody was stopped 3 minutes later. In initial labelling experiments, 25 µl of fixing solution (0.16 M $Na_2S_2O_3$ and 0.012 M KI) was added to stop the reaction. The supernatant was removed and 4 washes of the labelled antibody-T-sorb complex were carried out with PBS. The anti-T was eluted off the T-sorb with 400 µl of 2% NH_4OH and then returned to physiological pH; (7.2 - 7.4) with saturated KH_2PO_4. The labelled antibody was then either concentrated on an Amicon^R concentrator or purified on a Sephadex G-25 M prepacked PD-10 column (Pharmacia, Sweden); both of which were pre-equilibrated with 1.5% bovine serum albumin (Sigma, St. Louis).

b) Nonspecific IgG Immunoglobulin

100 µg of purified rabbit IgG immunoglobulin was labelled with ^{131}I (iodination grade, AECL) also by the iodine monochloride method. The protein was mixed with 3 µl Na ^{131}I and 1 ml 0.5 M phosphate buffer for 30 seconds. The reaction

was initiated upon the addition of 25 µl of a 1:50 dilution of the stock ICl solution and terminated 3 minutes later with 25 µl fixing solution. The labelled protein was separated by gel filtration on a Sephadex PD-10 column eluted with PBS.

c) Peanut Lectin and $F(ab')_2$ Nonspecific IgG

300 µl of PNA (0.3 mg/ml in PBS) was buffered with 20 µl of 0.5 M phosphate buffer, pH 7.4, in a 3 ml Reacti-Vial. Following the addition of 3-6 µl Na^{125} (3.7 MBq/µl, iodination grade, AECL), the reaction was initiated by adding 30 µl of Chloramine T (1 mg/ml in 0.05 M phosphate buffer, pH 7.4). The reaction was terminated 30 seconds later, by the addition of 60 µl sodium metabisulfite solution (1.2 mg/ml in 0.05 M phosphate buffer). In biodistribution studies utilizing $F(ab')_2$ simultaneously with PNA, 100 µl (1 mg/ml) of each protein solution was placed in 0.3 ml Reacti-Vials. The $F(ab')_2$ fragment and PNA were then labelled with I-131 and I-125, respectively, as outlined above. A Bio-Gel P-6DG column was used to remove free iodide.

3. Separation of Unreacted Free Iodide

a) Gel Filtration Media

i) Sephadex G-25 in PD-10 columns

Disposable prepacked PD-10^R columns of Sephadex G-25 medium were obtained from Pharmacia Ltd. and used for purification of radiolabelled proteins in anti-T studies. These columns have a bed volume of about 9 ml and a void volume for proteins of about 2.5 ml.

ii) Bio-Gel P-6DG

Bio-Gel P-6DG^R (Bio-Rad Lab, Mississauga, Ont.) is a desalting polyacrylamide gel with an exclusion limit of about 6000 caltons. The gel was hydrated by incubating it overnight at room temperature in PBS. Twice as much PBS was used as the expected packed volume of 8 ml/gram Bio-Gel P-6DG. The column used in initial PNA experiments was a PD-10 column from which the Sephadex was removed. To improve resolution, a

longer column, of about 11 ml packed bed volume, was prepared in a Pharmacia^{R} 9 mm x 300 mm column.

b) The Separation Procedures

To prevent nonspecific adherence, 0.5 ml of 1% BSA in PBS was passed through the columns before application of radiolabelled proteins. The protein mixture (antibody or lectin) was layered on top of the bed surface and allowed to drain in, before PNS was added to wash the sample into the bed. The eluting PBS was added at a flow rate of about 0.4 - 0.5 ml per minute. The eluate was monitored for both protein and radioactivity. A flow-through ultraviolet photometer (LDC Duo Monitor) was set at 280 nm to detect protein absorbence in comparison to the PBS buffer in a paried UV cell. The thin polyethylene cannula, carrying the eluate, also passed across the face of a shielded 3" x 3" NaI(Tl) crystal detector attached to a single channel analyzer. A dual-pen Fisher Recordall 5000 recorder was used to record the simultaneous detection of radioactivity and protein optical density.

4. Determinations of Labelling Yield and Radiochemical Purity

a) Trichloroacetic Acid (TCA) Precipitation

Aliquots of reaction mixtures were taken before and after purification procedures and diluted to 1 ml with 1% BSA in PBS. Following the addition of 1 ml of 20% TCA, the denatured protein mixture was mixed on a vortex mixer and then centrifuged at 500 rpm for 10 minutes. After removal of the supernatant, the precipitate was redispersed in 20% TCA before a second centrifugation (500 rpm x 10 minutes) was performed. The initial amount of radioactivity added had been assayed and following separations, the activity in the supernatants and the precipitate was determined in an automatic gamma well-counter.

b) Instant Thin Layer Chromatography (ITLC)

Ten or twenty micro-litre aliquots from the reaction mixtures and purified protein were spotted on silica gel

instant thin layer strips and dried. The chromatograms were developed to approximately 15 cm in 85% methanol in water and then cut into 1 cm strips and assayed for radioactivity on a Beckman 8000 gamma spectrometer.

c) Gel Filtration Fractionation

A radioactivity elution profile was obtained during the gel filtration separation of unreacted radioiodide from radiolabelled protein. This provided information on the relative distribution of radioactivity in the reaciton mixture. Analysis was carried out by either determining the area under the elution profile curves or by totalling the radioactivity of the fractions composing the salt or protein peaks. Nonspecific losses of radioactivity on the column were assumed to be loss of labelled protein, rather than free iodide.

5. Radiochemical Stability of Iodinated Peanut Lectin

The rate of hydrolysis of I-125 from radiolabelled peanut lectin was determined. Fifteen µg of PNA protein was labelled by the iodine monochloride method utilized for the radioiodination of IgG (as described in section C2b) and 15 µg was labelled by the Chloramine-T method (section C2c). Free unreacted iodide was removed from both labelled products by gel filtration with a polyacrylamide Biogel^{R} P-6DG (Bio-Rad Lab, Richmond, CA) - packed 9 mm x 300 mm column which had been pre-equilibrated with 1.5% BSA. Protein fractions were collected in BSA precoated falcon tubes and then stored at 4°C.

Three 100 µl samples of each labelled protein were taken 0, 1, 2, 3, 4, 5, 6, 7, 10, 14 and 17 days following radioiodination and mixed with 900 µl 1.5% BSA in PBS. One ml of 20% trichloracetic acid (TCA) was added to precipitate the protein, followed by centrifugation (500 rpm x 10 min.).

The initial mixture and the separated precipitate and supernatant were analyzed for radioactivity on a Beckman 8000 gamma counter employing the I-125 dpm program. The percentage of radioactivity which was TCA precipitated was calculated by

dividing the counts in the precipitate by the sum of activity in the precipitate and supernatant.

D. Tumor Cell Culturing

1. Preparation of Tissue Culture Media

Five hundred ml of sterile fetal bovine serum was heat-inactivated, by a one hour water bath incubation at 57°C, and then aseptically aliquoted into 50 ml samples before storage at -20°C. RPMI 1640 medium (500 ml) with L-glutamine and 25 mM HEPES buffer was aseptically supplemented with a 50 ml aliquot of heat inactivated fetal calf serum and 5 ml of 200 mM L-glutamine. The tissue culture media were stored at 4°C and used within 3 months.

2. Tumor Cell Culturing Procedures

All tumor culture procedures were carried out in a laminar flow hood which had been previously exposed to germicidal UV light and scurbbed with 70% isopropyl alcohol.

Suspension cultures of the mouse tumor cell lines RI lymphoma, EL4 lymphosarcoma and BW5 lymphoma were maintained in a carbon dioxide incubator at 37°C and a CO_2 concentration of 4 - 5%. To determine cell concentrations, aliquots were aseptically withdrawn from the cell suspensions and spotted onto a prepared hemocytometer and cells were counted at 150 X microscopic magnification. In viability determinations, trypan blue was added to the culture aliquot before microscopic examination and the percentage of cells which excluded dye was calculated.

The mouse tumor cell lines were subcultured every 2 or 3 days. Tissue culture media was preheated to 37°C in a serological water bath. Cells ($1 - 5 \times 10^5$) were inoculated into 10 ml of preheated culture media contained in 25 cm^2 sterile tissue culture flasks. The cells were recultured before a maximum cell concentration of 10^6 cells/ml was reached, in order to ensure log phase growth. Disposable serological borosilicate pipettes which were utilized for subculturing, were im-

mersed in a bleach solution following use and culture flasks and tubes were autoclaved before disposal.

E. In Vitro Tests of the Biological Activity of Radiolabelled Proteins

To prevent nonspecific adherence and protein losses in in vitro studies, tubes were pre-coated with bovine serum albumin (BSA). Three ml aliquots of 3% BSA in PBS solution were added to each 12 x 75 mm plastic tube, followed by incubation for one hour at 37°C and then for an additional 24 hours at 4°C. The BSA solution was removed and the tubes rinsed once, with 3 ml PBS. Tubes were capped and stored at 4°C until use. All binding studies were done in tubes precoated with BSA.

1. Studies with Neuraminidase-Treated Red Blood Cells N'RBC

a) Neuraminidase treatment

The T antigen in human red blood cells was exposed by neuraminidase treatment. Red blood cells were collected from the defribinated blood of a group O, rhesus negative donor and used within 2 days of collection. A 2% (V/V) suspension of packed red blood cells in PBS was incubated for 45 minutes at 37°C with Vibrio cholerae neuraminidase (Gibco Ltd.), at a final concentration of 5 units/ml. Saline replaced neuraminidase treatment, cells were washed by centrifugation (4x) with PBS and resuspended to a concentration of 2% (V/V).

b) Hemagglutination titre to N'RBC

Serial dilution, of radiolabelled and unlabelled lectin, were carried out with PBS in BSA-precoated tubes. Equal volumes (100 µl) of diluted lectin and N'RBC were mixed and incubated at 4°C for 30 minutes. The mixtures were then centrifuged (500 rpm x 5 minutes), gently resuspended and examined visually for hemagllutionation.

c) Binding studies to N'RBC

One ml of 2% RBC, or serial dilutions of a 2% suspension, and one ml of control RBC were placed in separate BSA-precoated tubes. The cells were incubated for 10 minutes at room temperature with 1% BSA in PBS. Following centrifugation, (500 rpm x 5 minutes), the supernatant was removed and 100 µl of diluted radiolabelled antibody or peanut lectin was added to the cell pellets. The mixtures were resuspended, counted for total radioactivity and incubated on ice for 30 minutes. Three washes were performed with 1% BSA in PBS before the final pellet was recounted for bound radioactivity.

The specificity of binding was assured by inhibition of binding in the presence of galactose. For these controls, both the 1% BSA preincubation step and the added radiolabelled protein contained 0.1 M galactose. The 1% BSA used for washing (post-^{125}I-PNA incubation) contained 0.02 M galactose.

2. Studies with Synthetic Carbohydrate Immunoadsorbents

Carbohydrate immunoadsorbents were obtained from Chem-Biomed Ltd. These "synsorbs" are composed of various mono-, di- or trisaccharides which are covalently linked by a 9 carbon 2-deoxy-3-0-(β-D-galactopyranosyl)-α-D-galacto pyranoside) immunoadsorbent was synthesized by the process of Ratcliffe et al (Carbohydr. Res., 93:35, 1981). The solid support of the immunoadsorbent was used as a control. 5 mg of both the T-sorb and synsorb blank were incubated with 0.5 ml 1.5% BSA for 10 minutes on BSA precoated tubes. Following removal of the BSA solution, 100 µl of diluted radiolabelled antibody or peanut lectin was added and the total content of radioacitvity was determined. The synsorb mixtures were incubated, with gentle rotation, at 4°C for one hour. Three washes were performed with 1.5% BSA in PBS before the immunoadsorbents were counted for bound radioactivity (I-125 dpm program Beckman 8000).

b) Study of carbohydrate-specificity of PNA

To determine the specificity of binding, immunoadsorbents prepared from other carbohydrates were incubated with I-125 PNA. Twenty mg of the various immunoadsorbents were weighed out into BSA precoated tubes. The synsorbs were incubated with 0.5 ml 1.5% BSA for 10 minutes, before 100 µl of ^{125}I-PNA (30 kBq/0.6 µg PNA in 100 µl PBS) was added.

The mixtures were incubated for 45 minutes at 4°C with continuous gentle agitation. The supernatant was removed and 3 washes of the synorbs were performed, using a 0.15% solution of BSA in PBS. Each synsorb was tested in triplicate. The bound radioactivity was determined on the Beckman 8000 gamma counter using the I-125 dpm program.

3. Binding to Tumor Cells

RI, EL 4 or BW5 cells, which excluded trypan blue in excess of 95% and were in the log pahse of growth, were used to test the binding of the radiolabelled antibodies or lectin. Aliquots containing 2 - 10 x 10^6 cells were added to BSA-precoated tubes along with 0.5 ml 1.5% BSA. The supernatant solution was removed following centrifugation (500 rpm x 5 min) and 100 µl of radiolabelled antibody or lectin was added to the pellet. The tumor cell pellets were resuspended in the radiolabelled protein solutions and the total added radioactivity was determined before the mixtures were incubated on ice for one hour. Cells were washed (3x) with tissue culture media before the final supernatant was removed and the pellet recounted for bound radioactivity. The specificity of binding was assured by inhibition of binding in the presence of galactose in a manner similar to the N'RBC cell studies.

F. Methods of Determining the In Vivo Biodistribution of Radiolabelled Antibodies and Peanut Lectin

1. Mouse Tumor Models

Six to eight week old male mice of the CBA/CAJ and C57-black strains, weighing generally between 20 and 25 grams, were housed in groups of 5 or 6 animals per cage and maintained on

standard laboratory chow (Wayne^{R} Lab-Blox, Chicago, IL) and tap water (ad libitum).

Tumor cells used for inoculation were determined to be greater than 95% viable by the trypan blue exclusion test. Following determination of the cell concentration, the suspension was centrifuged at 500 rpm for 5 minutes and resuspended in culture media, to the required concentration. The desired number of cells, generally 2 - 5 x 10^5 cells, was drawn up in syringes, in an aseptic environment, in the laminar flow hood. The mice were inoculated with a 0.1 or 0.2 ml subpannicular injection, in the dorsal region. RI lymphoma cells were used in CBA/CAJ mice and EL4 lymphosarcoma cells were used in 57/Black mice.

Tumors were allowed to grow for 10-14 days, before the animals were utilized for biodistribution or imaging studies of the radiolabelled protein. Lugol's solution (5 drops per 200 ml) was added to the drinking water commencing 2 days prior to injection of the radiolabelled product and continued until termination of the studies.

2. Biodistribution Studies in Tumor Bearing Mice

Tumor bearing mice were injected i.v., via the the tail vein, with 0.1 or 0.2 ml of the radioiodinated antibodies (anti-T specific or nonspecific IgG, intact or $F(ab')_2$ fragment) and/or peanut lectin. In dual label experiments, the nonspecific protein (IgG; intact or $F(ab')_2$) was labelled with I-131 and the specific protein (anti-T IgG or peanut lectin) was labelled with I-125). In single label experiments, the I-125 isotope was used.

The relative amount of radioactivity injected into each animal was determined by counting the syringes before and after injection, on a whole body counter. Three extra doses were prepared in syringes and counted on a whole body counter before and after being used to prepare 1:50 dilutions. Aliquots of 0.5 ml of these dilutions served as injection standards to cor-

relate injection dose to the radioactivity determined for tissue samples.

The mice were etherized and then sacrificed by cardiac puncture/exsanguination at time periods such as 3, 8, 24 and 48 hours following injection. The tissues of interest were excised in their entirety, blotted free of blood and were weighed directly in tared plastic counting tubes. The remaining carcass, tail (injection site), a portion of the trachea with the thyroid, were also placed in counting tubes but not weighed.

Samples, along with the diluted injection standards, were assayed for radioactivity on a programmable automated NaI gamma well counter. In studies involving only a ^{125}I-labelled protein, the coincidence method was used for absolute determination of ^{125}I radioactivity. In dual label experiments, a spill-over correction was employed to correct for counts occurring in the ^{125}I window due to ^{131}I radioactivity.

The percentage of radioactivity per gram tissue or entire organ was calculated on the basis of the injected activity and as a percentage of the activity remaining in the body at time of dissection. Tissue ratios such as tumor:blood and tumor:muscle were calculated on a per weight basis.

a) ^{125}I-PNA in CBA/CAJ mice with RI tumors

Male CBA/CAJ mice of about 25 g weight were inoculated s.p. in the right flank with 5×10^5 RI lymphoma cells. Eleven to fifteen days following inoculation, the tumor-bearing mice received a 0.2 ml caudal i.v. injection of 50 kBq ^{125}I-PNA (185 kBq/µg). Between 7 and 8 mice were sacrificed at each time period of 3, 8, 24 and 48 hours following injection and analyzed for I-125 radioactivity as described earlier.

b) Paired-label studies

i) Anti-T vs. IgG in CBA/CAJ mice with RI tumors

CNA/CAJ mice were inoculated s.p. with 6×10^5 cells over the right flank, 9 days prior to use in biodistribution studies. Anti-T antibodies were labelled, as described,

to a specific activity of 2 MBq/µg with I-125 and concentrated on an Amicon Concentrator. TCA precipitation of the protein solution revealed that 91.5 ± 1.3% of the radioactivity was protein bound. Nonspecific IgG antibodies were labelled to a specific activity of 7.5 MBq/µg with I-131 and purified on a Sephadex PD-10 column. 99.11 ± 0.06 of an aliquot of the desalted preparation was precipitated during TCA analysis. Mice were injected i.v. with a simultaneous injection of 10 MBq anti-T and 8 MBq IgG. Animals were dissected at 2, 4, 8, 24 and 48 hours following injection. Tissue samples were analyzed for ^{125}I and ^{131}I radioactivity.

ii) PNA vs. F(ab')$_2$ in CBA/CAJ mice with RI tumors and C57-BL mice with EL4 tumors

CBA/CAJ mice were inoculated with 5 x 10^5 RI cells and C57 black mice were inoculated with 6 x 10^5 EL4 cells, 10-12 days prior to biodistribution studies. A 0.2 ml i.v. tail injection containing 3 MBq ^{125}I-PNA (165 kBq/µg) and 3.5 MBq ^{131}I-F(ab')$_2$ IgG fragment (150 kBq/µg) was given to the tumor bearing mice. TCA precipitation and ITLC (85% methanol in water) analysis revealed that 96.12 ± 1.32% and 95.92 ± 1.49% of the I-131 and I-125, respectively, was protein bound. Four to six mice were dissected at each of the time periods of 24 and 48 hours for the biodistributuon studies in CBA/CAJ - RI bearing C57/Bl-EL4 bearing mice. Additional CBA/CAJ mice were dissected at 8 and 72 hours. The samples and standards were analyzed for I-131 and I-125 radioactivity as described earlier.

3. Analysis of Plasma Samples from Biodistribution Studies

When the biodistribution study blood samples were taken by cardiac puncture, an aliquot was also placed in 1.5 ml Eppendorf^R centrifuge tubes. Following centrifugation (12,800 rpm x 2 min), a 200 µl aliquot of plasma was transferred to another Eppendorf centrifuge tube and 200 µl of 20% trichloroacetic acid (TCA) was added, mixed on a vortex-mixer and centrifuged. The radioactivity in the precipitated protein and supernatant was determined with a Beckman 8000 gamma counter.

4. Whole Body Gamma Camera Imaging

CBA/CAJ mice were injected with 330 kBq ^{125}I anti-T intact IgG or 350 kBq ^{125}I-PNA and anaesthetized with Nembutal^{R} 10 minutes prior to imaging. A PhoGamma IV (Searle) with a pin-hole collimator was calibrated with an ^{125}I standard. Each mouse was secured with masking tape to a positioning board and serial images of the posterior view (15,000 - 20,000 counts) were obtained at time periods between 3 and 72 hours post-injection of the radiolabelled proteins.

A. Tumor Cell Lines and Animal Tumor Model

In order to study the in vivo localizing capability of T antigen-avid proteins, it was desirable to have an appropriate animal tumor model. Reproducible moderate size tumors, which are rapidly produced with a vascular supply similar to naturally occurring tumors, would provide an ideal tumor model, if the cells expressed the T antigen.

A number of mouse tumor cell lines were tested by direct and indirect immunofluorescence, for the binding of T antigen-avid reagents. An RI lymphoma mouse cell line was shown to bind T-affinity purified human and rabbit antibody and peanut lectin.

The RI radiation-induced lymphatic leukemia was initially isolated from CBA(H-2^K) mice and has been passaged in mice as the ascitic form and grown in vitro as a tissue culture cell line by several workers. This cell line grows as a suspension in vitro, thereby facilitating cell quantitation, animal inoculation and cell viability determinations. Since the cells grow singly in suspension (although with a slight tendency to clump), trypsin treatment of the cells was not required prior to mice inoculation. This was an important parameter for the animal model, since trypsin is knowm to cleave off cell surface glycoproteins, such as the T antigen and is cell toxic.

As a radiation-induced tumor, this lymphoma may provide a more suitable animal model for spontaneous T antigen-bearing cancers in humans than a chemically or virally induced tumor. Those animal tumors which are induced by carcinogens or viruses, often express many neo-antigens and this results in an artifactual immunogenicity and antigenicity, not usually seen in spontaneous and most radiation-induced tumors.

The tumor cell line had been passaged for an extended period of time as an in vitro tissue culture and this may possibly allow selection of cells adapted for in vitro growth, rather than growth in animals. To determine if the RI leukemic

cells would grow in vivo, as a solid subpannicular tumor which could be readily imaged and dissected, CBA/CAJ mice were inoculated with 1×10^5, 2×10^5, 5×10^5 and 10×10^5 viable tumor cells. Solid, palpable tumors were evident, for all doses, 5 to 8 days following injection, with the largest doses generally resulting in tumor masses ranging from 150 mg to 1500 mg. Larger tumors tended to have necrotic centers and showed wide ranges of degree of vascularization. In further experiments, the CBA/CAJ mouse-RI tumor model was used 9-12 days following the s.p. inoculation of 2×10^5 to 6×10^5 viable tumor cells.

Another murine mouse tumor line, utilized for in vivo and in vitro studies, was the EL4 lymphoid tumor which was isolated in 1945 from C57 black ($H\text{-}2^b$) mice, following treatment with 1:10 dimethyl 1:2 benzanthrene and it has been maintained by tissue culture and serial i.p. and s.p. transmissions. The EL4 cells were found to not bind T antigen-avid antibodies and lectins and this cell line was used as a control for both in vivo and in vitro studies with the RI cells. Mice, of the strain C57/Bl, were inocultaed with 5×10^5 cells, and tumors, with gross appearances similar to RI tumors, were ready for biodistribution studies 10-12 days later.

The BW-5147 is a lymphocytic leukemia, originated in an AKR mouse ($H\text{-}2^K$) in 1954, which has been maintained by i.p. and s.p. passages and in vitro culturing, was used as a non-T antigen expressing control for the RI cells. Attempts to grow this tumor in CBA mice (with the same major histocompatibility classification as AKR) were unsuccessful, probably due to minor histocompatibility differences.

B., Preliminary Studies with Anti-T and Nonspecific IgG Antibodies

1. Modifications of the Radioiodination Procedures

The initial labelling procedure, involved preincubation of 100 µg of anti-T antibody with 10 mg T sorb in order to pro-

tect the binding site. Ther reaction mixture, in 500 µl borate buffer, was gently agitated for 2 hours at 4°C within a 3 ml vial. A centrifugation step (5 min at 800 rpm) was followed by 6 washes (2 ml borate buffer, pH 7.2) in order to remove antibody which was not T-sorb-bound. The protected antibody was mixed with 15-20 MBq $Na^{125}I$ in 1 ml borate buffer for 30 seconds, followed by the addition of 6.86 mg ICl in 100 µl 2N NaCl. The vial was agitated on a vortex mixer for 2 minutes and the reaction was stopped by the addition of 100 µl fixing solution (0.016 M $Na_2S_2O_3$ and 0.012 M KI). Unreacted iodine was removed by withdrawal of the supernatant and 5 subsequent 2 ml washes to the T-sorb with PBS. The radioiodinated protein was eluted from the T-sorb with 500 µl of 1% NH_4OH and then adjusted to pH 7.2 with KH_2PO_4 (16.5% solution).

This procedure was systematically modified in subsequent experiments to optimize the retention of biological activity of the radioiodinated product. Most of the modifications were made to decrease mechanical or chemical damage to the protein during the T-sorb incubation and iodination steps. Mechanical or chemical damage may alter the quarternary or tertiary structure of the protein, which may lead to changes in the binding affinity of the protein for T antigen-containing substances.

Centrifugation of the reaction mixture to isolate the T-sorb-protein complex was found to be unnecessary. The immunoadsorbent matrix (silylaminated, calcined, diatomaceous earth) was of sufficiently high density to separate efficiently by gravity alone. Six washes were initially performed on the antibody-T sorb complex prior to labelling. The number of washes was reduced to three, since the rabbit anti-T had already been purified on T-sorb. The vigorous vortexing, initially carried out during iodination procedures, was changed to gentle hand agitation.

The borate buffer was replaced by a phosphate buffer because it was suspected that the borate ion may interact with either the antibody or the T-antigen immunodeterminant disaccharide. Parallel synthesis of ^{125}I-anti-T, using either borare or phosphate buffer, resulted in similar labelling efficiencies (40-43%) and desorption capabilities of the anti-T (47-51%). Thus, subsequent iodinations were conducted in phosphate buffer.

The relatively high ICl/protein ratios, used initially to ensure high labelling efficiencies, exposed the protein to higher concentrations of the oxidizing agent than were necessary. The molar ratio of ICl was decreased four-fold, so that the maximum iodine incorporation would be about 1.5 atoms per antibody molecule, based on 100% chemical yield.

The chemical exposure of the protein was also reduced by elimination of the reducing agent (sodium metabisulfite). Since the antibody is immobolized on the T-sorb particles, the oxidizing reagent and free I^+ and were simply removed with the supernatant and four washes of the T-sorb protein complex. Duplicate reactions, in which the reducing step was omitted in one of the reactions, revealed that a higher hemagglutination titre to N'RBC was obtained for the antibodies labelled without reduction. The binding of the nonfixed antibody preparation was 4.7% to N'RBC and only 0.3% to control RBC. Once the antibody is radioiodinated on the T-sorb, a desorption agent is required to elute the labelled antibody from the immunoadsorbent.

In the methodology developed, an increased pH, with the use of 1% NH_4OH, was utilized to elute antibody from the T-sorb. To evaluate the effects of various concentrations of NH_4OH for antibody desorption, two labelling experiments were carried out in which aliquots of the labelled antibody-T sorb complexes were desorbed with either 0.5%, 1% or 2% NH_4OH. The desorption capability was found to be highest for the 2% NH_4OH, which eluted 79-85% of the T-sorb-associated radioactivity, as com-

pared to 70-74% and 54-57% with the 1% and 0.5% concentrations, respectively. The radioiodinated antibody preparations, eluted by all three NH_4OH concentrations, showed similar binding to T-sorb (∿70%) and N'RBC (∿5%). A 2% NH_4OH concentration was therefore selected, due to its greater desorption capabilities. Other methods of desorption, including 0.1 M KCl pH 2.2 for 30 seconds or 3 minutes, 0.4 borate buffer for 1 minute, or 1% acetic acid for 1 minute, were all found to be less effective than 2% NH_4OH in eluting the labelled antibody from the T-sorb.

Nonspecific adsorption of the antibody to contact surfaces was a very signigicant problem throughout any of its manipulations. Losses as high as 25% on Falcon^R tubes, 85% in an Amicon^R B-15 concentrator and 50% on a Sephadex PD-10 column, were observed. It was, therefore, desirable to precoat every surface, that would come in contact with the antibody, with a 1% BSA solution to decrease the nonspecific adsorption losses. Therefore, insufficient washings of the T-sorb was unlikely to be the source of free iodide.

A blank labelling experiment, in which phosphate buffer replaced the protein, indicated that 25% or the total radioactivity used in the reaction remained associated with the T-sorb and reacti-vial after washing the T-sorb. The NH_4OH desorption process then eluted 38% of the radioactivity on the T-sorb and reacti-vial. The source of free iodide in the antibody preparations, therefore, appeared to be nonspecific adsorption and desorption of I^- from the reacti-vial and T-sorb. A purification technique, such as gel filtration was therefore needed to remove the 6-10% free iodide from the anti-T preparations following desorption.

The final modified labelling procedure for the anti-T rabbit IgG and nonspecific rabbit IgG is described in sections C2a and C2b, respectively. The protected T-sorb iodine monochloride iodinations of anti-T resulted in labelling efficien-

cies which ranged from 25-50% and the direct iodination of IgG gave iodination efficiencies of 50-80%.

2. Biodistribution Studies and In Vitro Binding

Preliminary biodistribution studies were carried out with ^{125}I-anti-T in RI lymphoma bearing CBA/CAJ mice. It was found that the ^{125}I-labelled anti-T localized in the tumors to some extent but that prolonged, elevated blood levels maintained a high background-tissue radioactivity. Three preliminary biodistribution studies indicated that average tumor:muscle and tumor:blood ratios of radioactivity at 25 hours (total of 10 mice) were 5.9 $\pm$ 0.5:1 and 0.57 $\pm$ 0.04:1, respectively. Preliminary gamma camera imaging analysis, of RI tumor-bearing mice injected i.v. with 300 kBq ^{125}I-PNA, revealed that some tumor localization was evident 4 days post-injection.

A complication, when determining the tumor localizing capabilities of an antibody or any protein preparation, is the nonspecific accumulation of macromolecules in tumors. Tumors often have a larger extravascular-extracellular space, greater blood vessel permeability and less efficient lymphatic drainage than found in normal tissue. Therefore, most radiolabelled proteins localize to some extent in tumors. The use of double isotopic labels, a technique first introduced by Pressman et al, Cancer Res., 17:845, 1957, is helpful in distinguishing between specific and nonspecific antibody localization in tumor and other tissues. Specific anti-tumor antibodies are labelled with one isotope of iodine, e.g., ^{125}I, and normal control globulins are labelled with another, such as ^{131}I. Simulataneous injection of the two preparations allows evaluation of the tumor for localization of specific antibody and control nonspecific protein.

This paired-label technique was therefore utilized to determine the specificity of anti-T tumor localization. Mice bearing RI tumors were simultaneously injected with 10 MBq

^{125}I-anti-T and 8 MBq ^{131}I-IgG. The localization of the antibodies in tissue samples was subsequently determined by differential radioactive analysis of the two isotopes present. Six mice were dissected at each time period of 2, 4, 8, 24 and 48 hours. The radioactivity, determined for ^{125}I-anti-T, was corrected for the spillover of ^{131}I counts occurring in the ^{125}I window. Biodistribution data was calculated as a percentage of the injected dose per gram of wet tissue or per intact organ and are presented in Table 1 and Table 2 for the 8, 24 and 48 hour time periods. Although not assayed in this particular biodistribution study, preliminary studies indicated less than 1% of the injected dose was contained in the lung at 25 lours.

TABLE 1

COMPARATIVE TISSUE BIODISTRIBUTION OF
I-125 ANTI-T AND I-131 NORMAL GAMMA GLOBULIN
IN RI LYMPHOMA-BEARING CBA/CAJ MICE

		(Percent Dose Per Gram Tissue)					
		8 Hr (N=6)		24 Hr (N=6)		48 Hr (N=6)	
		T-AB	IgG	T-AB	IgG	T-AB	IgG
Tumor	%Dos/Gram	4.67	7.31	4.08	7.14	2.74	5.08
	Stan.Dev.	1.23	1.93	0.81	1.71	2.74	5.08
Blood	%Dos/Gram	11.65	20.43	8.10	15.88	5.50	11.11
	Stan.Dev.	2.30	4.72	0.79	2.01	0.92	2.38
Liver	%Dos/Gram	3.22	4.21	2.49	3.69	1.62	2.41
	Stan.Dev.	0.62	1.03	0.22	0.37	0.30	0.64
Spleen	%Dos/Gram	3.01	4.57	2.17	3.78	1.64	2.92
	Stan.Dev.	0.80	1.26	0.23	0.52	0.40	0.75
Kidney	%Dos/gram	4.59	6.22	3.95	5.47	2.46	3.71
	Stan.Dev.	1.07	1.69	0.67	1.08	0.44	0.86
Muscle	%Dos/Gram	0.66	1.02	0.78	1.37	0.63	1.16
	Stan.Dev.	0.22	0.33	0.17	0.29	0.27	0.38

Footnote: T-AB = Anti-T antibodies; IgG = Nonspecific Normal Gamma Globulin

TABLE 2

COMPARATIVE TISSUE BIODISTRIBUTION OF
I-125 ANTI-I AND I-131 NORMAL GAMMA GLOBULIN
IN RI LYMPHOMA-BEARING CBA/CAJ MICE

		(Percent Dose Per Entire Organ) 8 Hr (N=6)		24 Hr (N=6)		48 Hr (N=6)	
		T-AB	IgG	T-AB	IgG	T-AB	IgG
Tumor	%Dos/Org.	1.91	3.01	1.51	2.60	2.06	3.60
	Stan.Dev.	1.20	1.94	0.74	1.24	1.00	1.66
Blood	%Dos/Org.	16.66	29.21	11.58	22.71	7.86	15.89
	Stan.Dev.	3.28	6.75	1.13	2.88	1.32	3.41
Liver	%Dos/Org.	4.16	5.42	2.93	4.34	1.91	2.87
	Stan.Dev.	0.74	1.21	0.22	0.39	0.32	0.80
Spleen	%Dos/Org.	0.32	0.49	0.24	0.42	0.17	0.30
	Stan.Dev.	0.12	0.18	0.05	0.07	0.03	0.04
Kidney	%Dos/Org.	1.59	2.15	1.24	1.81	0.82	1.25
	Stan.Dev.	0.35	0.52	0.17	0.32	0.15	0.38

Footnote: T-AB = Anti-T antibodies; IgG = Nonspecific Normal Gamma Globulin

The ratio of radioactivity for tissue:blood and tissue: muscle, are also presented in Table 3 and were calculated on a gram to gram basis. These ratios show the relative concentrations in the various tissues and may give an indication of the potential usefulness in imaging studies.

TABLE 3

COMPARATIVE TISSUE RATIOS OF RADIOACTIVITY OF I-125 ANTI-T ANTIBODY AND I-131 NORMAL GAMMA GLOBULIN IN RI LYMPHOMA-BEARING CBA/CAJ MICE

		8 Hr (N=6)		24 Hr (N=6)		48 Hr (N=6)	
		T-AB	IgG	T-AB	IgG	T-AB	IgG
Blood	Organ/Blood	1.00	1.00	1.00	1.00	1.00	1.00
	Stan.Dev	0.00	0.00	0.00	0.40	0.00	0.00
	Organ/Muscle	18.47	20.60	10.64	11.87	9.65	10.34
	Stan.Dev.	3,42	3.05	1.70	2.17	3.05	3.36
Liver	Organ/Blood	0.28	0.21	0.31	0.23	0.30	0.22
	Stan.Dev.	0.02	0.01	0.01	0.02	0.03	0.02
	Organ/Muscle	5.14	4.24	3.28	2.76	2.78	2.20
	Stan.Dev.	1.12	0.64	0.56	0.48	0.70	0.63
Spleen	Organ/Blood	0.26	0.22	0.27	0.24	0.30	0.26
	Stan.Dev.	0.06	0.04	0.04	0.03	0.06	0.04
	Organ/Muscle	4.86	4.68	2.89	2.85	2.84	2.69
	Stan.Dev.	1.78	1.35	0.71	0.71	0.93	0.88
Kidney	Organ/Blood	0.39	0.30	0.49	0.36	0.45	0.34
	Stan.Dev.	0.04	0.02	0.08	0.06	0.07	0.04
	Organ/Muscle	7.21	6.22	5.28	4.35	4.21	3.38
	Stan.Dev.	1.24	0.93	1.56	1.30	0.92	0.86
Tumor	Organ/Blood	0.40	0.36	0.50	0.45	0.49	0.44
	Stan.Dev.	0.08	0.07	0.08	0.07	0.07	0.09
	Organ/Muscle	7.44	7.51	5.43	5.41	4.87	4.73
	Stan.Dev.	2.38	2.42	1.51	1.72	1.98	2.09
Muscle	Organ/Blood	0.06	0.05	0.10	0.09	0.12	0.11
	Stan.Dev.	0.01	0.01	0.02	0.02	0.05	0.05
	Organ/Muscle	1.00	1.00	1.00	1.00	1.00	1.00
	Stan.Dev.	0.00	0.00	0.00	0.00	0.00	0.00

From the organs dissected, and analyzed for ^{125}I-anti-T, only the kidneys and tumor were found to have tissue:blood ratios of radioactivity larger than 0.30:1 at any of the time periods evaluated. The ^{125}I-anti-T tumor:muscle ratio was 7.4:1 at 8 hours, 5.4:1 at 24 hours and 4.9:1 at 48 hours. The tumor:blood ratio remained at 0.5:1 for both the 24 and 48 hour time periods. Preliminary biodistribution studies revealed tumor:blood ratios at 24 hours of 0.4:1 and 0.15:1 for lymph nodes and thymus, respectively.

The IgG preparation was found to have a greater whole body and tissue retention than the specific anti-T. The percentage of injected dose of ^{131}I-IgG, per gram of tissue, was found to be 1.5-2 fold higher in most tissues as compared to anti-T. At five days post-injection, blood levels were an average of 4 fold higher for the nonspecific antibody than for specific anti-HCG. These differences were not observed in non-tumor bearing mice.

Although the absolute tissue retention of the IgG was elevated, relative tissue ratios such as tumor:muscle and tumor:blood were very similar to those observed for anti-T. At 24 hours, the tumor:muscle ratio was 5.1:1 and the tumor:blood ratio was 0.45:1 for the ^{131}I-IgG. Similar tumor:blood ratios have been ovserved when radiolabelled nonspecific IgG was injected in tumor bearing rats.

An informative manner for the analysis of the specificity of antibody localization, is to calculate the specificity or localization index. The ratios of relative concentrations such as tissue:blood, obtained for the specific antibody are divided by the corresponding factor, calculated independently for the control protein. The specific:nonspecific ratio can be standardized by ratios in the blood, injected doses, liver, lung or muscle.

In this study, the blood was chosen to analyze the data, since 1) poor blood clearance is one of the major complications

in antibody studies and 2) nonspecific macromolecular tumor localization is primarily due to a changed vasculature in the tumor tissue. The ratios were, therefore, calculated using the percentage of injected dose per gram of blood or tissue in the following equation:

$$\text{specificity index} = \frac{\text{tissue:blood anti-T-IgG}}{\text{tissue:blood nonspecific IgG}}$$

When this ratio is much greater than one for the tumor, but not other tissues, it indicates that the increased antibody radioactivity in the tumor is probably due to specific tumor tissue binding in addition to hypervascularization and interstitial fluid accumulation in the tumor. The specificity index of the radioiodinated anti-T for the tumor was 1.11 to 1.13 at the time periods of 8 to 48 hours and was not found to be significantly different from the remainig carcass or other tissues. Although most other tissues had a specificity index of 0.96 to 1.14 at 48 hours, the value from the tumor data was 2.34. This indicated localization in the tumor, which nonspecific accumulation could only account for about 43% of the tumor retention. In the anti-T/IgG study, approximately 90% of the localization in the tumor, and most other tissues, is probably due to nonspecific localization. This seems to indicate that the anti-T is not specifically retained by interaction with the T antigen on the RI cells. Metabolism of the antibody may be occurring before it is fixed on the cell surface, since the tumor receives such a small proportion of the cardiac output.

Results of the in vitro binding are displayed in Table 4 and indicate the anti-T bound relative avidly to the T immunoadsorbent, but the binding was not extremely high to N'RBC (which by definition of the T antigen, contains a large number of T antigen receptors). The in vitro tumor-cell binding did not indicate a high specificity of the radiolabelled anti-T for antigens expressed on the RI cells.

TABLE 4

IN VITRO BINDING OF ANTI-T AND NORMAL IgG*

	Anti-T	Normal IgG
Binding to T-sorb (specific)	45 - 85% (n=8)	0.8 - 0.9% (n=4)
Binding to Synsorb (control)	2.5 - 10% (n=4)	2.1 - 2.2% (n=4)
Binding to N'RBC (specific)	3 - 8.2% (n=4)	0.20 - 0.22% (n=2)
Binding to RBC (control)	0.3 - 3.3% (n=4)	0.28 - 0.3% (n=2)
Binding to RI (specific)	1% (n=1)	0.9% (n=1)
Binding to EL4 (control)	0.6% (n=1)	0.8% (n=1)

* - Anti-T and IgG were radiolabelled with I-125 and I-131, respectively.

- Values are expressed as percentage of added radioactivity bound to ligands following incubation and subsequent washings.
- The number of independent determinations is given in parenthesis.

The anti-T was prepared by injecting rabbits with the synthetic β-D-Gal(1→3)α-D-GalNAc conjugated by an 8 carbon linking chain to carrier BSA molecules. Antibodies, which were specific for the linking arm or any of the many antigenic sites present on a BSA molecule, could be produced along with the antibodies specific for the T disaccharide. Affinity purification on the T immunoadsorbent should remove anti-BSA antibodies but antibodies specific for the linking carbon chain would still be present in the "purified" antibody preparation. Therefore, only a proportion of these "anti-T" antibodies would also have a high affinity for the natural antigen, exposed on the cell surface.

It was desirable to carry on these studies with a homogeneous monoclonal antibody preparation with a very high specificity for the T antigen. However, due to the sophisticated techniques involved in preparing a monoclonal antibody with a highly avid and specific binding ability, a source of monoclonal anti-T was not available to continue the antibody studies at the time.

Another T antigen-avid protein, the peanut lectin, was available as a homogeneous, affinity-purified protein. Initial *in vitro* binding studies indicated high binding affinities for not only the T immunoadsorbent but also membrane-bound T specificities in N'RBC and RI tumor cells.

C. Peanut Lectin

1. Reconstitution and UV Spectroscopic Analysis of PNA

The peanut lectin, utilized in *in vitro* and *in vivo* studies, was obtained from EY Laboratories (San Mateo, CA) and the product specifications indicated a single band by polyacrylamide-disc gel electrophoresis. The hemagglutination titre of the PNA lot obtained was 1:2048 to neuraminidase-treated red blood cells. The protein was kept frozen in the lyophilized form until portions of 3 to 5 mg were reconstituted with phosphate buffered saline, pH 7.4 (PBS). The reconstituted protein

was passed through a sterile 0.22 µm filter and maintained under aseptic conditions at 4°C.

The reconstituted protein was analyzed by UV spectroscopy at 280 nm for both qualitative and quantitative analysis. The absorption spectra revealed a double maxima at 277 nm and 283 nm and also a small shoulder peak at 290 nm. These peaks are believed to be due to aromatic amino acid side chains such as tryosine. Quantitative increases of the peaks, in the 270-300 nm range, occur during interactions of PNA with specific saccharides and glycoproteins.

Several PNA preparations were accurately reconstituted to 1 mg/ml and the optical density was measured at 280 nm. Six separate determinations of the extinction coefficient at 280 nm resulted in a value of 8.45 ± 1.26 for the $E^{1\ mg/ml}_{280\ nm}$ of PNA. Quantitative determinations of the peanut lectin concentration were calculated using the equation $A = E^{1\ mg/ml}_{1\ cm}\ c.l$ (where A = absorbence, c = concentration in mg/ml and l = path length in centimeters). The $E^{1\ mg/ml}_{1\ cm}$ extinction coefficient used in these determinations was the published value at 280 nm of 9.6. The reconstituted peanut lectin solutions were stored at 4° at concentrations of 1 mg/ml and used within 2 months or discarded.

2. Quality Control of Radioiodine ($Na^{125}I$) Solution

a) Radionuclidic purity of $Na^{125}I$

The reagent grade, no-carrier added radioiodine ($Na^{125}I$), used for labelling procedures, was tested for both radio-chemical and radionuclidic purity. A NaI(Tl) well crystal and multichannel analyzer were used to determine the energy spectrum of $Na^{125}I$. Two photopeaks were observed, which occurred at about 28 keV and 55 keV and the gamma spectrum observed correlated well with a published spectra of I-125. The photopeaks are due to the single emissions at 27.4 keV and 35.5 keV, and the coincidence peaks which occur at 55 and 63 keV. No peaks were observed at higher energies.

b) Radiochemical purity of $Na^{125}I$

Instant thin layer chromatography (ITLC) was used to analyze the radiochemical purity of the radioiodine. Aliquots of the $Na^{125}I$ were spotted on silica gel ITLC strips and developed by ascending chromatography (85% methanol in water), to determine the presence of any iodate. In this system, the I^- migrates with the solvent front, with typical Rf values of 0.9, while the radioiodate (IO_3^-) remains close to the origin with an Rf value about 0.2. Analysis of the strips revealed that the radioiodide peak accounted for 97.6% of the total radioactivity. Thus, this product was acceptable for radioiodination purposes.

3. Radioiodination and Radiochemical Purification of PNA

The chloramine-T method was utilized for the radioiodination of peanut lectin. The $Na^{125}I$ qas buffered initially to pH 7.4 with phosphate buffer to prevent protein damage from the alkaline pH of the radioiodine solution and to optimize the pH for the radioiodination. The ratio, on a weight basis, of chloramine-T to protein, 0.3:1, was chosen to provide a consistent labelling efficiency while minimizing exposure of the protein to extensive oxidizing conditions. The sodium metabisulfite was added in excess (twice the molar quantity of chloramine-T) in order to terminate the reaction and reduce unreacted I^+ to I^-.

The radioiodination efficiency was assessed by three different methods; TCA precipitation, silica gel ITLC and gel filtration chromatography. Radiolabelling efficiencies were routinely determined by TCA precipitation and generally correlated well with the values determined by ITLC and for analysis of the elution profiles. The labelling efficiencies of 9 separate radioiodinations are presented in Table 5. An average labelling efficiency of 58.2 $\pm$ 6.5% was attained with a range of 50-74% based on TCA precipitation analysis of the labelled protein, the unbound ^{125}I migrated at the solvent front (typical Rf values of 0.90 to 0.95) while the protein fraction re-

mained essentially at the origin (Rf values of -0.5 to 0.15). If the samples, which were analyzed, contained a very large amount of free iodide, the estimated value of unbound radioactivity was generally higher when determined by ITLC analysis, than by TCA precipitation. For example, analysis of one radiolabelled protein/radioiodide mixture revealed 83% free iodide by ITLC and 76% free iodide by TCA precipitation. This is possible due to nonspecific trapping of the free iodide in precipitated carrier BSA protein. Addition of carrier KI during TCA precipitation may possibly help rectify this problem.

TABLE 5

ANALYSIS OF LABELLING EFFICIENCY IN CHLORAMINE-T RADIOIODINATIONPROCEDURES OF PEANUT LECTIN

Experiment	Percent Labelling Efficiency as determined by: *		
	TCA Precipitation	Gel Filtration Elution Profile	Silica Gel ITLC 85% MeOH/H_2O
1	50.1 ± 0.3 (n=2)	48.8%	-
2	64.5 ± 4.9 (n=2)	59.8%	-
3	58.2 ± 0.8 (n=2)	56.2%	-
4	60.0 ± 0.5 (n=4)	57.0%	-
5	65.1 ± 0.3 (n=2)	64.9%	-
6	66.8 ± 0.9 (n=2)	67.2%	-
7	51.8 ± 2.0 (n=3)	50.3%	49.2 ± 1.3 (n=3)
8	49.8 ± 1.6 (n=3)	52.2%	49.4 ± 2.2 (n=3)
9	57.1 ± 1.0 (n=2)	56.1%	55.2 ± 0.8 (n=3)

* n = number of determinations, all gel filtration calculations are based on one determination from the desalting procedure.

Separation of unreacted radioiodine was carried out by gel filtration chromatography. A polyacrylamide gel, BioGel P-6DG, was chosen, since the media is designed for desalting procedures and exhibits low nonspecific adsorption of macromolecules. Initially, the BioGel P-6DG media was packed into 5 ml syringe barrels or emptied PD-10 columns (Pharmacia), and did not quite provide baseline resolution between the void volume (protein peak) and the salt peak (iodide). Therefore, longer columns were packed [9 mm x 300 mm (Pharmacie)] with bed volumes of about 11 ml. These longer columns provided excellent baseline resolution between protein and salt peaks.

A column eluate was analyzed, in sequence, for both protein optical density (at 280 nm) and radioactivity. The protein absorbence peak was usually noted to appear at an elution volume of 2.5 ml and fractions containing the complete absorption peak were collected in about a 3 ml volume. Generally, the first 1.5 ml of protein collected was utilized for futher in vivo and in vitro studies and this fraction generally contained in excess of 80 percent of the protein radioacitvity. A further 1 to 1.5 ml was eluted at baseline radioactivity before the radioiodide salt peak occurred. Analysis of the radioactivity elution profile provided additional information on the labelling efficiency of the radioiodination which correlated well with analysis by TCA and/or ITLC techniques. Between 92 and 99.5% of the protein-associated radioactivity added to the column, was recovered in the protein peak fractions. TCA precipitation analysis of the two major radioactivity elution peaks revealed that 96-97% of the radioactivity was precipitated in the protein peak while only 5-7% of the radioactivity precipitated in the iodide peak. Therefore, the BioGel P-6DG column provided an efficient means of removing free iodide from the radiolabelled lectin and sample dilutions of the protein fractions, as a result of this desalting procedure, were usually only 3 59 5 fold.

4. Radiochemical Stability of ^{125}I-Peanut Lectin

The radiochemical stability of radioiodinated PNA was determined for ^{125}I-PNA prepared by two different radioiodination techniques; the routinely used Chloramine-T method, and also the iodine monochloride method. Both preparations were desalted separately on the same P-6DG column and the purified protein mixtures were stored at 4°C, pH 7.4 in BSA-precoated tubes.

These radioiodinated protein solutions were analyzed by TCA precipitation to determine the percentage of protein-bound radioactivity. The I-125 PNA, labelled by the Chloramine-T method, was somewhat more stable than that produced by the ICl technique. During the initial first few days following radioiodination, the daily loss of radioiodine was more rapid than that observed after the first week. At two weeks, 2.2 and 3.3% of the radioiodide had hydrolyzed from the Chloramine-T- and ICl-radioiodinated PNA, respectively, and about 65% of this hydrolysis had already occurred in the initial 4 days.

Overall, the rate of hydrolysis of radioiodine from ^{125}I-PNA is relatively slow, when the radiolabelled PNA is stored at 4°C, and a pH of 7.4.

5. In vitro Binding Studies of ^{125}I-PNA

The biological binding affinity of radiolabelled peanut lectin was determined by various *in vitro* studies. The presence of a large number of Thomsen-Friedenreich receptors on neuraminidase-treated red blood cells, N'RBC, makes these cells extremely useful ligands to test the binding affinity of PNA.

The biological activity of a lectin can be estimated by comparing the end point hemagglutination (the largest dilution of cells showing macroscopic evidence of agglutinated cells). Serial dilutions of N'RBC were carried out and the hemagglutination titre of both the labelled and unlabelled lectin were carried out in parallel. The end point titre occurred at a concentration of 0.5 µg/ml for both the labelled and unlabelled

product. Although the subjectiveness of the hemagglutination only allows this method to provide a rough estimate of biological activity, it appears as if the radiolabelling procedure does not seriously affect the biological affinity of PNA.

The routine method of testing the biological activity of radioiodinated peanut lectin is to test the percentage binding to one ml of a 2% suspension of neuraminidase-treated red blood cells (approximately 1.5×10^8 cells). The results of the experiments, measuring the binding of ^{125}I-PNA to N'RBC are presented in Table 6 and represent ten separate radioiodinations of PNA and twelve subsequent binding studies. Normal untreated red blood cells served as controls, for they have been shown to contain less than 3% of the number of receptors found in N'RBC.

TABLE 6

IN VITRO BINDING OF RADIOIODINATED PEANUT LECTIN

(Percentage Bound*)

Ligands	T-Antigen Expressing		Control	
	N'RBC	N'RBC + Galactose	Control RBC	
Human Erythrocytes (1.5×10^8 cells)	66 ± 9 (50 to 84) n=12	2.1 ± 0.7 (1.5 to 2.8) n=3	1.2 ± 1.2 (0.2 to 3.8) n=11	
	RI	RI + Galactose	EL4	BW5-147
Tumor Cells (5×10^6 cells)	20 ± 3 (16 to 25) n=7	1.9 ± 0.9 (1.0 to 2.8) n=2	0.5 ± 0.4 (0.2 to 1.0) n=5	0.27 ± 0.15 (0.41 to 0.4) n=4
	T-sorb	T-sorb + Galactose	Blank Synsorb	
Immunoadsorbents (5 mg)	22 ± 3 (17 to 27) n=5	3.1 ± 1.2 (1.9 to 4.3) n=2	1.6 ± 0.7 (0.9 to 2.4) n=3	

* Values are expressed as a percentage of added radioactivity ^{125}I-PNA bound (following incubations and subsequent washes) in the form of mean ± s.d. ( ) range, and n = number of independent replicates.

The radioiodinated peanut lectin was found to be very avid for binding to N'RBC with an average binding affinity of 66%. Although preliminary studies with anti-T antibodies had indicated maximum percentages of binding to N'RBC of 8.2%, the peanut lectin showed binding capabilities as high as 84% in some cases. Since almost every N'RBC binding determination was carried out with a newly prepared ^{125}I-PNA and a freshly neuraminidase-treated lot of red blood cells, there was some degree of variation in the N'RBC binding. The specificity of the PNA-N'RBC interaction was assured by the relative lack of PNA binding to N'RBC (2.1%) in the presence of galactose. Control red blood cells also failed to show any significant binding, with an average value of 1.2%.

The in vitro binding of ^{125}I-PNA was tested with another membrane-associated glycoprotein ligand, the RI tumor cells. As indicated in Table 6, the peanut lectin was shown to have a very high binding affinity for RI cells, with an average 20 percent bound. In another binding determination, utilizing 1×10^7 cells rather than 5×10^6 tumor cells, 39 $\pm$ 0.3% (n=3) of the ^{125}I-PNA remained bound to the cell pellet following 4 PBS washes. The specificity of binding was indicated by a 90 percent inhibition of binding in the presence of galactose. The EL4 tumor cell line was used as a control for tumor cell binding, averaging 0.4%, was observed for EL4 cells as illustrated in Table 6. Another control tumor cell line, BW5-147, was also found to have a very low binding affinity for PNA. Four separate radiolabelled peanut lectin preparations revealed an average percentage binding of only 0.27 $\pm$ 0.15% to the BW-147 murine tumor cell line.

The various in vitro binding results of PNA indicated a specific and preferential binding of this lectin for cells and immunoadsorbents bearing T-like antigens.

6. Carbohydrate Binding Specificity of ^{125}I-PNA

Traditional methods of analyzing specific reactions of lectin and antibodies usually involve a comparison of the inhibitory ability of various mono- and oligosaccharides for either hemagglutination or glycoprotein precipitation. Erythrocyte membrane glycoproteins, and many soluble glycoproteins, appear to be very complex and are capable of binding a wide range of lectins with various saccharide-binding specificities. Synthetic immunoadsorbents, prepared by covalently linking various mono-, di- and trisaccharides, via a 9-carbon linking arm to silylaminated, calcined, diatomaceous earth particles, would contain only the particular carbohydrate desired. By studying the binding of I-125 labelled peanut lectin to various synthetic immunoadsorbents, it is possible to delineate a few of the carbohydrate binding specificities of this lectin. The final percentage of ^{125}I lectin bound to each synsorb, was determined following the 45 minute incubation and the subsequent PBS washes to remove any unbound lectin. The carbohydrate immunoadsorbents which were tested, their specific activities (μM carbohydrate per gram immunoadsorbent), and the ^{125}I-PNA binding results are all summarized in Table 7. The binding data are presented in 3 forms. The percentage of added ^{125}I-PNA which was bound to the carbohydrate-synsorbs was determined in triplicate and corrected for nonspecific binding by subtracting the binding which occurred with a blank immunoadsorbent (no attached sugar residues). Since all of the synsorbs inhibited various degrees of sugar incorporation, all of the binding data was standardized for specificity inter-comparisons, by calculating the percentage bound per 0.1 μM carbohydrate. Finally, galactose was assigned a binding ability of 1.0 and the binding abilities of all other sugars were calculated relative to galactose on a per μM basis.

TABLE 7

CARBOHYDRATE BINDING SPECIFICITY
OF ^{125}I-PNA USING SYNTHETIC IMMUNOADSORBENTS

Carbohydrate Specificity and μM/gram of Immunoadsorbent	Percentage* Bound per 20 mg Synsorb	Percentage Bound per 0.1 μM Carbohydrate	Binding** Relative to Galactose
β-Gal (4.52 μM/g)	8.623 ± 0.657	0.191	1.00
β-Glc (0.28 μM/g)	0.326 ± 0.112	0.116	0.61
α-GalNAc (1→3)β-Gal (0.3 μM/g)	< 0	< 0	< 0
β-Gal(1→3)β-GlcNAc (0.48 μM/g)	0.676 ± 0.105	0.141	0.74
β-Gal(1→3)β-GalNAc (0.58 μM/g)	42.61 ± 1.510	7.346	38.47
β-Gal(1→3)α-GalNAc (0.27 μM/g)	25.50 ± 2.880	9.444	49.44
β-GalcNAc(1→3)β-Gal(1→4)β-Glc (0.35 μM/g)	2.205 ± 0.301	-.630	3.30

* Corrected for cpm bound to blank immunoadsorbent mean ± one s.d. of 3 individual determinations

** Relative binding ability standardizing to a galactose-binding ability of 1.0.

In this study, the binding ability of the synthetic T-immunoadsorbent, was found to be 49.4 relative to galactose. This relative binding occurred when the disaccharide β-D-Gal-(1→3)-GalNAc was covalently linked in the α position to the bridging arm of the synsorb. A lower binding ability of 38.5 was found when the disaccharide was covalently bound by a linkage to the bridging arm. A better inhibitory ability was found for the synthetic carbohydrate chains when the T determinant was attached via an α, rather than β linkage to the β-O-position of N-tosyl-L-serine.

Of the other carbohydrates tested, none showed a binding ability exceeding 10% of that found between PNA and the T determinant. For weakly bound carbohydrates, where nonspecific interactions may contribute significantly to binding, wider fluctuations in the affinity are often found between one specificity study and another.

The importance of the configuration of the C-4 hydroxyl in the penultimate pyranose is evident when comparing the relative binding of PNA to β-Gal(1→3)D-GlcNAc and β-Gal(1→3)D-GalNAc. By substituting glucose for galactose as the penultimate sugar, the binding decreased from 49.4 to 0.74. The study of binding specificity of a lectin by simply measuring the percentage binding to synthetic immunoadsorbents appears to offer an efficient rapid means of analysis that produces results similar to those seen with the accepted inhibition studies.

A very specific interaction of PNA with the T-immunodeterminant disaccharide was evident in this study and supports the in vitro tumor and N'RBC cell binding studies. Therefore, CBA/CAJ mice with RI tumors were utilized to test the potential of radioiodinated peanut lectin for the radioimmunodetection of cancer.

7. Biodistribution of ^{125}I-PNA in CBA/CAJ Mice Bearing RI Tumors

The peanut lectin, used in this biodistribution study, was radioiodinated with an efficiency of 60% to provide a specific activity of 185 kBq/µg (∿0.26 atoms iodine/molecule). In vitro analysis of this particular preparation of ^{125}I-PNA revealed that 66.6 ± 1.2% bound to a one ml 2% suspension of N'RBC and 39% of the radioactivity was bound to 1×10^7 RI tumor cells. Retention of biological activity following radioiodination was demonstrated by a preserved hemagglutination titre to N'RBC. Over 98 percent of the radioactivity in the injected product could be precipitated by trichloroacetic acid. The CBA/CAJ male mice had received a subpannicular inoculation of 5×10^5 RI cells, in the right flank. This resulted in well defined solid tumors, averaging 250 mg, at 11 to 15 days post-inoculation. Lugol's solution was added to the animals' drinking water, commencing two days prior to i.v. injection. The tumor-bearing mice received a 0.2 ml caudal i.v. injection of 50 kBq ^{125}I-PNA. At 3, 8, 24 and 48 hours following injection, the mice were sacrificed by cardiac puncture/exsanguination under light ether anaesthesia. The liver, spleen, kidneys, pancreas and tumor were dissected as entire organs. The stomach, duodenum (first 2 cm of GIT) and remaining GIT were assayed with their contents. The blood sample content of radioactivity was extrapolated to the entire blood pool on the basis of animal body weight (entire blood pool = 6.5% body weight), and the skeletal muscle sample was taken from the hind thigh contralateral to the tumor site. The tissue samples, along with injection standards, were assayed for radioactivity in an automated gamma spectrometer utilizing the coincidence method for absolute determination of ^{125}I radioactivity.

The results of the biodistribution study are presented as a percentage of injected ^{125}I-PNA per gram wet tissue and on the basis of entire organ accumulation in Tables 8 and 9, re-

spectively. The values are given as the mean and standard deviation of the 6 to 8 mice sacrificed per time period.

TABLE 8

TISSUE DISTRIBUTION OF I-125
IN CBA/CAJ MICE BEARING RI TUMORS

(Percent Dose Per Gram Tissue)

		3HR N=7	8HR N=6	24 HR N=8	48 HR N=8
Tumor	%Dos/Gram	3.894	2.524	0.618	0.178
	Stan.Dev.	1.073	1.151	0.283	0.064
Blood	%Dos/Gram	1.543	0.926	0.124	0.011
	Stan.Dev.	0.453	0.590	0.058	0.003
Liver	%Dos/Gram	0.748	0.441	0.140	0.064
	Stan.Dev.	0.155	0.231	0.021	0.009
Spleen	%Dos/Gram	4.799	2.706	0.765	0.191
	Stan.Dev.	0.560	0.439	0.118	0.042
Kidney	%Dos/Gram	8.931	3.849	0.917	0.323
	Stan.Dev.	3.757	1.453	0.145	0.079
Stomach	%Dos/Gram	8.880	8.468	1.030	0.075
	Stan.Dev.	1.457	6.199	0.611	0.072
Pancreas	%Dos/Gram	0.977	0.562	0.058	0.009
	Stan.Dev.	0.192	0.365	0.024	0.002
Duodenum	%Dos/Gram	2.495	1.043	0.146	0.018
	Stan.Dev.	0.785	1.432	0.059	0.005
GIT	%Dos/Gram	1.432	0.854	0.082	0.012
	Stan.Dev.	0.332	0.490	0.025	0.003
Muscle	%Dos/Gram	0.339	0.145	0.024	0.005
	Stan.Dev.	0.086	0.110	0.013	0.002

TABLE 9

TISSUE DISTRIBUTION OF I-125
IN CBA/CAJ MICE BEARING RI TUMORS

		(Percent Dose Per Intact Organ)			
		3HR N=7	8HR N=6	24 HR N=8	48 HR N=8
Tumor	%Dos/Org.	0.572	0.557	0.095	0.077
	Stan.Dev.	0.285	0.316	0.051	0.056
Blood	%Dos/Org.	2.759	1.621	0.220	0.020
	Stan.Dev.	0.814	1.028	0.114	0.006
Liver	%Dos/Org.	0.976	0.611	0.188	0.101
	Stan.Dev.	0.173	0.289	0.034	0.011
Spleen	%Dos/Org.	0.640	0.403	0.105	0.028
	Stan.Dev.	0.110	0.089	0.014	0.005
Kidney	%Dos/Org.	3.543	1.570	0.343	0.125
	Stan.Dev.	1.347	0.493	0.060	0.029
Stomach	%Dos/Org.	2.724	2.938	0.343	0.039
	Stan.Dev.	0.742	1.471	0.199	0.011
Pancreas	%Dos/Org.	0.062	0.074	0.011	0.001
	Stan.Dev.	0.038	0.063	0.005	0.000
Duodenum	%Dos/Org.	0.299	0.234	0.018	0.002
	Stan.Dev.	0.085	0.183	0.009	0.001
GIT	%Dos/Org.	3.717	2.156	0.193	0.033
	Stan.Dev.	0.791	1.079	0.059	0.006

The biodistribution data reveal that most tissues exhibited a relatively rapid elimination of ^{125}I-PNA, although the tumor retention was greater than most other tissues. One gram of blood contained only 0.01% of the injected activity at 48 hours and this was less than one percent of the radioactivity initially present at 3 hours. The tumor, on the other hand, retained at 48 hours, almost 5 percent of the radioactivity initially present at 3 hours, so that one gram of tumor tissue contained 0.62% and 0.18% of the injected ^{125}I-PNA at 24 and 48 hours, respectively. On a gram basis, the only tissues which exhibited a consistently greater uptake of ^{125}I-radioactivity than the tumor, were the kidney and spleen. An initially high stomach uptake of ^{125}I decreased rapidly with time so that only 0.8% of the 3 hour stomach radioactivity was retained at 48 hours. The liver, pancreas and muscle were found to have a relatively low uptake of ^{125}I-PNA.

The peanut lectin demonstrated a very rapid whole body elimination. The percentage of injected radioactivity remaining in the mouse, at time of sacrifice, was only 42 $\pm$ 13%, 29 $\pm$ 15%, 4.4 $\pm$ 1.6% and 1.3 $\pm$ 0.4% at 3, 8, 24 and 48 hours, respectively. The blood clearance was also very rapid, as indicated by the biodistribution data. At 8 hours only 5.4 $\pm$ 0.7% of the radioactivity remaining in the body was contained in the entire blood pool and by 48 hours this had fallen to 0.8 $\pm$ 0.4%.

The rapid blood clearance, when combined with a tumor retention greater than that of most other organs, resulted in very favorable tumor-to-background ratios. Tissue:blood and tissue:muscle ratios are presented in Table 10.

TABLE 10

TISSUE RATIOS OF RADIOACTIVITY OF I-125 PEANUT LECTIN IN CBA/CAJ MICE BEARING RI LYMPHOMA TUMORS

		Organ to Blood Ratios			
		3HR N=7	8HR N=6	24HR N=8	48HR N=8
Blood	Organ/Blood	1.00	1.00	1.00	1.00
	Stan.Dev.	0.00	0.00	0.00	0.00
Liver	Organ/Blood	0.50	0.52	1.26	6.17
	Stan.Dev.	0.06	0.12	0.41	1.33
Spleen	Organ/Blood	3.30	3.92	6.90	19.02
	Stan.Dev.	0.88	2.13	2.24	8.07
Kidney	Organ/Blood	5.78	5.06	8.40	31.31
	Stan.Dev.	1.35	1.94	2.90	9.92
Stomach	Organ/Blood	6.03	8.88	8.04	6.71
	Stan.Dev.	1.22	1.34	1.40	1.14
Pancreas	Organ/Blood	0.66	0.62	0.48	0.81
	Stan.Dev.	0.14	0.10	0.08	0.20
Duodenum	Organ/Blood	1.68	1.98	1.29	1.63
	Stan.Dev.	0.64	0.78	0.61	0.22
GIT	Organ/Blood	0.95	0.96	0.70	1.17
	Stan.Dev.	0.16	0.16	0.13	0.21
Muscle	Organ/Blood	0.23	0.15	0.19	0.46
	Stan.Dev.	0.07	0.04	0.06	0.10
Tumor	Organ/Blood	2.57	3.30	5.79	17.01
	Stan.Dev.	0.61	1.82	3.17	5.74

TABLE 10 (cont)

TISSUE RATIOS OF RADIOACTIVITY OF I-125 PEANUT LECTIN IN CBA/CAJ MICE BEARING RI LYMPHOMA TUMORS

		Organ to Muscle Ratios			
		3HR	8HR	24HR	48HR
Blood	Organ/Muscle	4.67	7.13	5.59	2.30
	Stan.Dev.	1.24	2.54	1.59	0.57
Liver	Organ/Muscle	2.26	3.72	6.87	14.06
	Stan.Dev.	0.39	1.62	2.23	4.08
Spleen	Organ/Muscle	14.62	27.80	37.43	41.58
	Stan.Dev.	2.21	17.16	11.58	13.20
Kidney	Organ/Muscle	26.62	36.67	46.02	70.21
	Stan.Dev.	9.70	22.75	17.07	21.45
Stomach	Organ/Muscle	27.58	63.05	44.69	15.55
	Stan.Dev.	7.69	22.08	14.80	5.20
Pancreas	Organ/Muscle	2.97	4.30	2.64	1.88
	Stan.Dev.	0.62	1.36	0.88	0.73
Duodenum	Organ/Muscle	7.40	14.07	6.88	3.77
	Stan.Dev.	1.93	6.42	3.03	1.06
GIT	Organ/Muscle	4.30	6.92	3.79	2.71
	Stan.Dev.	0.63	2.91	0.82	0.91
Muscle	Organ/Muscle	1.00	1.00	1.00	1.00
	Stan.Dev.	0.00	0.00	0.00	0.00
Tumor	Organ/Muscle	11.54	22.44	33.60	39.80
	Stan.Dev.	2.62	10.66	20.80	17.59

Average tumor:blood ratios of radioactivity increased steadily as a function of time. A much more significant retention of radioactivity in the tumor than in the blood resulted in an average tumor:blood ratio of 17:1 at 48 hours. The tissue:blood ratios of ^{125}I-PNA are compared for the tumor and various other organs as a function of time in RI lymphoma bearing CBA/CAJ mice. A steadily increasing tumor:blood ratio occurred as a function of time. Metabolism of the radioiodinated peanut lectin resulted in production of free iodide and the in vivo biological breakdown of ^{125}I-PNA was found to be very rapid. Trichloroacetic acid precipitation of the plasma samples revealed the presence of 70% free iodide in the plasma at 3 hours and 80% after 8 hours. This was reflected in the organ distribution, in that the stomach had maximum relative uptake at 8 hours. The important role that the kidney plays in the elimination of free iodide probably leads to the high kidney:blood ratio. Muscle:blood ratios were very low throughout the study period.

Another background tissue, used for comparison of tissue radioactivity, was the skeletal muscle, and the tumor:muscle ratio also increased steadily as time progressed. Average tumor:muscle ratios that were already 13:1 at 3 hours, reached an average value of 40:1 at 48 hours with individual ratios as high as 65:1. The liver:muscle ratio is generally only 30% of the radioactivity ratio shown by tumor:muscle.

Since the radioactivity was determined for the reaming carcass and for the tissues-of-interest, it was possible to calculate the percentage of radioactivity in various tissues, based on the radioactivity remaining in the body at the time of sacrifice. The percentage of remaining dose per gram of tissue showed that at 3 and 8 hours, the tumor contained about 10% of the remaining body radioactivity, per gram of tumor tissue. By 24 hours, the overall elimination of radioactivity from the whole body was greater than that from the tumor. Such selective tumor retention resulted in 16% of the remaining body

radioactivity being contained per gram of tumor tissue at 24 and 48 hours post-^{125}I-PNA-injection. Thus, there was a very low muscle retention of ^{125}I-PNA and a blood clearance of radioactivity which was faster than whole body elimination.

The amount of radioactivity contained in various entire organs at 24 hours, expressed as a percentage of the ^{125}I-PNA remaining in the body, was as follows: tumor (2.6 $\pm$ 1.6%), liver (4.7 $\pm$ 1.4%), spleen (2.6 $\pm$ 0.7%), both kidneys (8.5 $\pm$ 2.5%), stomach (8.0 $\pm$ 3.8%), pancreas (0.26 $\pm$ 0.12%), small and large intestine (4.7 $\pm$ 1.4%). Preliminary biodistribution studies had revealed that the thymus, brain, fat and bone showed very low uptake of radioiodinated peanut lectin.

The serial gamma camera images reflected the tissue biodistribution data. Posterior views of 20,000 counts, collected with a Pho Gamma IV camera (pinhole collimator) at 6, 24, 48 and 72 hours showed a diffuse whole body distribution on the initial day with some localization evident in the tumor. The stomach, left kidney and spleen, which all lie in close proximity and in fact overlie one another in the posterior view, combine to produce a "hot spot" on the left side of the animal. Rapid clearance from the blood, stomach and muscle and a relatively slower clearance in the tumor, allowed an even clearer delineation of the tumor by 24 and 48 hours. The tumor shows the most intense localization in the body at 48 and 72 hours. No blood pool subtraction was necessary for successful imaging.

Dissection of an animal imaged at 72 hours revealed that 37% of the total radioactivity remaining in the body was contained within the tumor. The tumor:muscle ratio in this particular mouse was 45:1 and the tumor:blood ratio was 7.5:1. The "hot spot" in the region of the neck corresponds to the salivary glands, which contained 5.6% of the total activity remaining in the body. Levels of radioactivity in other organs at this time were: thyroid 1%, stomach 2.7%; kidneys 3.5%, lungs 1.6%and testes plus seminal vesicles 1.2%.

Although localization of the radioactivity in the kidney, stomach and salivary gland was probably due to the radioiodide from the metabolized ^{125}I-PNA, there may also be some ^{125}I-PNA specific binding in these tissues. The peanut lectin has been used *in vitro* for the histochemical staining of mouse organ sections and PNA reactive glycoproteins have been found in the stomach, salivary glands, GIT, kidney, lung, pancreas and sex organs. However, results of *in vitro* binding to tissue sections are not easily extrapolated to *in vivo* localization studies. Tissue sections reveal sites not normally available to a macromolecule. The intracellular cytoplasm and Golgi zones are readily available in tissue slices, for interaction with a protein molecule. The final sialic acid-capping reaction, in the sequential synthesis of normal cell surface glycoproteins, occurs within the Golgi apparatus. The T antigen is believed to be a precursor of normal cellular glycoproteins, and as such would be expected to be found, in the unmasked form, within the secretory bodies and Golgi apparatus of normal cells. These sites are not normally available for macromolecular interaction in intact cells, but are exposed in tissue sections. Indeed, one of the major sites of PNA interaction in histochemical studies of mouse tissues was found to be the Golgi zones and stored secretory bodies.

Peanut lectin reactive sites have also been found, in *in vitro* studies, at extracellular locations. The mucous secretions and liminal surface of epithelial linings in the GIT, pancreatic and genitourinary tracts have been found to bind PNA when mouse tissue sections were examined *in vitro*. When tissue sections of human breast tissue were examined for *in vitro* binding of PNA, they also demonstrated a PNA-binding pattern which tended to be confined to luminal surfaces of epithelial cells and their secretions.

Although these sites are readily available for peanut lectin interaction when tissue sections are tested *in vitro*,

the liminal surfaces are essentially "outside" the body and are usually immunologically privileged and poorly vascularized. The poor contact with blood and lymph in these locations may not allow the in vivo localization and binding of a macromolecule, such as radioiodinated peanut lectin, before the protein is metabolized.

The importance of the vascularity and macromolecular accessibility of a tissue is demonstrated in the present invention by the failure of in vivo localization of radioiodinated PNA in the thymus of CBA/CAJ mice. Although 65 to 90 percent of mouse thymocytes have been found to bind PNA when tested histologically or in cell suspension only 0.003% of the injected dose of ^{125}I-PNA was found in the thymus, 24 hours post-injection. The thymus possesses a specialized vasculature, with an especially thick basement membrane and epithelial cell layer. The epithelial membrane acts as a barrier to impede passage of macromolecules, such as the peanut lectin, to the PNA reactive cells in the parenchymal interior.

The vascularity of the tumor also plays an important role in the accessibility of tumor cell surface antigens for interaction with radiolabelled lectins and other protein macromolecules. Animal tumor models, with solid subcutaneous tumors, provide tumor antigens which are located outside the host's normal vascular bed and do not allow fpr extremely rapid antigen-lectin interaction. When this is borne in mind, the clear tumor images obtained at 24 and 48 hours and tissue biodistribution data indicate a localization and retention of the radiiodinated peanut lectin which is possibly only limited by the tumor vascularity.

The selective tumor retention and fast blood clearance of radioiodinated PNA, combine to produce tumor:blood ratios such as 17:1 and these results are excellent when compared to other proteins and antibodies used for the radioimmunodetection of cancer. The localization of tumors using affinity purified

anti-CEA (carcinoembryonic antigen) has been complicated by low tumor:blood ratios of, for example, 1.3:1 at 24 hours and 2.4:1 at 48 hours. The absolute requirement of computerized blood pool subtraction for successful imaging with anti-CEA has led some workers to express doubts for its routine clinical usefulness. A high blood background may also complicate studies utilizing human anti-T immunoglobulins. These antibodies are primarily of the IgM class and, therefore, are largely confined to the bloodstream.

The solid subpannicular RI tumors, used in studying the biodistribution of ^{125}I-PNA, were analyzed for presence of the T antigen following in vivo growth and they were found to lose the antigen to some extent. When grown in vitro in tissue culture media as a cell suspension, 70 to 90 percent of the RI tumor cells were found to bind peanut lectin, as well as a human and rabbit preparation of anti-T. The binding of anti-T antibodies to RI tumor cells was detected by indirect immunofluorescence.

Following 11 to 17 days growth in vivo as a solid subpannicular tumor, only between 14 and 18% of the cells expressed the T antigen, independent of the tumor mass. Therefore, the expression of the PNA binding receptors on the tumor cells appears to be sensitive to the environment in which the cells grow.

Changes in cell surface glycoproteins have been documented for other tumor cell lines. The TA3-Ha ascites mammary adenocarcinoma cells have been shown to shed T- and MN antigens from the cell surfaces and during transfer of the ascites form, to either suspension culture or a solid form of in vivo tumor, the tumor cells were found to lose cell surface glycorpoteins. The glycoprotein content of the 13762 rat mammary adenocarcinoma also appears to depend on the environment or on the condition of growth. The major sialoglycoprotein, ASPG-1, of the ascites form of 13762 rat tumor cells has been shown to contain

PNA receptors. The solid 13762 tumor, from which all the ascites sublines were derived, does not contain ASPG-1, as detected by PNA. When this tumor cell line is grown in the ascites form, the ASPG-1 glycoprotein appears to be shed from the cell surface and soluble ASPG-1 glycoprotein is found in the ascites fluid and plasma of ascites-tumor-bearing rats.

Considering the localization of ^{125}I-PNA was relatively high in an animal tumor model in which only 14 to 18% of the cells of the tumor cell mass express PNA receptors, radiolabelled peanut lectin appears to present great potential as an agent for the radioimmunodetection of cancer.

8. The In Vivo Specificity of Tumor Localization of ^{125}I-PNA

In order to gain a better insight into the mechanism of in vivo PNA localization in RI tumors, it was desirable to determine the relative contribution of specific and nonspecific uptake in animal tumor models. The specificity of in vitro binding to RI tumor cells was demonstrated by the fact that addition of galactose produced an inhibition of ^{125}I-PNA binding to RI tumor cells in culture and by the absence of binding when tumor cells, EL4, not expressing the T antigen were tested.

Although antibodies within a certain class, e.g., IgG, of different binding specificities, are sturcturally similar, a nonspecific lectin is not readily available to act as a control for studies of peanut lectin binding. Therefore, a protein molecule of similar molecular weight, $F(ab')_2$ nonspecific rabbit IgG fragment, was chosen. This fragment has a molecular weight of 100,000 to 105,000 and is prepared from IgG molecules by cleavage of the Fc portion of IgG, with pepsin treatment. The Fc fragment is involved in the binding of complement, tissues and macrophages, so that its removal in the preapration of $F(ab')_2$ fragments will hopefully decrease nonspecific accumulation in both normal tissues (lack of Fc binding) and tumors (lower molecular weight).

The $F(ab')_2$ nonspecific rabbit IgG fragment, therefore, was chosed as a control protein to be labelled with I-131 and the injected simultaneously with ^{125}I-PNA. Differnetial radioactive counting of tissue samples for the two different iodine radioisotopes then allows for analysis of the specificity of PNA localization.

Two animal tumor mdoels were used to analyze the specificity of tumor localization. The EL4 mouse lymphoid tumor cell line does not express receptors for the peanut lectin and when tested in vitro, radioiodinated peanut lectin had very low affinity for this tumor cell line (Table 6). Therefore, 6×10^5 viable EL4 cells were injected s.p. in C57 black mice, to provide a solid subpannicular tumor 10-12 days later. The C57/Black mice, bearing EL4 tumors, then served as a control animal-tumor model for the CBA/CAJ mice, bearing RI cells.

In separate biodistribution studies, both sets of tumor-bearing animals were injected simultaneously with 3 MBq ^{125}I-PNA (165 KB]/µg) and 3.5 MBq ^{131}I-$F(ab')_2$. Between 4 and 6 animals in both groups of tumor-bearing mice were dissected at the time periods of 24 and 48 hours. Additional CBA/CAJ mice were sacrificed at 8 and 72 hours. The tissue samples and injection standards were analyzed for I-131 and I-125 radioactivity, as described earlier.

The specificity of the protein localization in the two different tumor animal models was analyzed by calculating the specificity indices. The ratio of relative tissue:blood radioactivity concentration obtained for radiolabelled peanut lectin was divided by the corresponding tissue:blood ratio for the control protein, as outlined in the following equation:

$$\text{specificity index} = \frac{\text{tissue:blood } (^{125}\text{I-PNA})}{\text{tissue:blood } (^{131}\text{I-F(ab')}_2)}$$

The numerical specificity indices are listed in Table 11. The percentage injected dose per gram of tissue (utilized in calculating these ratios) and the percentage injected dose incorporated per entire organ are outlined in Tables 12 and 13, respectively.

TABLE 11

LOCALIZATION INDEX OF PEANUT LECTIN

$$\frac{\text{Tissue:Blood } {}^{125}\text{I-Peanut Lectin}}{\text{Tissue:Blood } {}^{131}\text{I-F(ab')}_2 \text{ IgG}}$$

	24 hr		48 hr	
	RI Tumor CBA/CAJ Mice n=4	EL4 Tumor C57/Bl Mice n=6	RI Tumor CBA/CAJ Mice n=5	EL4 Tumor CBA/CAJ Mice n=4
Tumor	11.5 ± 1.1	1.3 ± 0.1	8.4 ± 1.2	1.6 ± 0.5
Muscle	2.5 ± 0.3	2.4 ± 0.3	2.7 ± 1.2	2.5 ± 0.5
Liver	4.1 ± 0.4	3.4 ± 0.3	5.4 ± 0.4	6.8 ± 1.3
Kidney	6.1 ± 0.4	6.3 ± 0.8	6.6 ± 0.3	9.9 ± 2.0
Stomach	2.9 ± 0.3	1.9 ± 0.5	3.4 ± 0.3	2.8 ± 0.4
GIT	2.4 ± 0.3	2.1 ± 0.1	2.7 ± 0.2	2.9 ± 0.2
Salvary	7.6 ± 1.1	3.0 ± 0.7	19.0 ± 1.6	3.5 ± 0.9
Sex Organs	3.6 ± 1.5	2.3 ± 0.4	6.2 ± 1.6	4.9 ± 1.8
Thyroid	3.4 ± 0.9	4.4 ± 2.5	3.1 ± 1.1	3.8 ± 2.8
Spleen	25.6 ± 2.7	6.2 ± 0.4	27.0 ± 2.0	8.7 ± 2.3
Lung	3.6 ± 1.5	2.9 ± 0.3	4.4 ± 0.3	4.6 ± 1.4
Thymus	2.0 ± 0.6	-	2.1 ± 0.3	-

TABLE 12

COMPARATIVE BIODISTRIBUTION OF I-125 PEANUT LECTIN AND I-131 $F(AB')_2$

PERCENT INJECTED DOSE INCORPORATED PER ENTIRE ORGAN

		CBA/CAJ Mice Bearing RI Tumors							
		8 HR(N=3)		24HR(N=4)		48HR(N=5)		72HR(N=1)	
		PNA	FAB	PNA	FAB	PNA	FAB	PNA	FAB
Blood	$\bar{X}$	1.88	8.13	0.30	1.35	0.11	0.39	0.04	0.16
	SD	0.48	0.86	0.03	0.17	0.01	0.05	0.00	0.00
Liver	$\bar{X}$	1.24	2.69	0.45	0.52	0.32	0.23	0.25	0.14
	SD	0.18	0.51	0.06	0.16	0.01	0.02	0.00	0.00
Spleen	$\bar{X}$	0.79	0.19	0.19	0.03	0.09	0.01	0.04	0.00
	SD	0.02	0.04	0.02	0.00	0.01	0.00	0.00	0.00
Kidney	$\bar{X}$	4.48	4.48	0.96	0.71	0.42	0.26	0.25	0.14
	SD	0.69	0.73	0.04	0.04	0.03	0.01	0.00	0.00
Stomach	$\bar{X}$	3.08	4.97	0.69	1.11	0.11	0.13	0.05	0.04
	SD	0.67	1.08	0.18	0.44	0.05	0.06	0.00	0.00
GIT	$\bar{X}$	4.01	5.63	0.50	0.94	0.16	0.24	0.08	0.10
	SD	0.66	0.54	0.14	0.20	0.01	0.01	0.00	0.00
Salivary	$\bar{X}$	1.10	1.47	0.23	0.14	0.20	0.04	0.14	0.01
	SD	0.56	0.84	0.02	0.04	0.00	0.01	0.00	0.00
Muscle	$\bar{X}$	0.16	0.28	0.02	0.04	0.00	0.01	0.00	0.00
	SD	0.03	0.05	0.00	0.00	0.00	0.00	0.00	0.00
Tumor	$\bar{X}$	1.33	0.41	0.09	0.13	0.03	0.03	0.01	0.00
	SD	10.6	0.27	0.28	0.05	0.25	0.08	0.00	0.00
Lung	$\bar{X}$	0.33	0.82	1.21	0.48	0.49	0.22	0.22	0.12
	SD	0.03	0.07	0.02	0.12	0.00	0.00	0.00	0.00
Sex	$\bar{X}$	0.79	1.05	0.08	0.10	0.05	0.03	0.97	1.24
	SD	0.35	0.42	0.04	0.06	0.00	0.00	0.00	0.00
Thyroid	$\bar{X}$	0.67	0.92	0.37	0.48	0.46	0.60	0.00	0.00
	SD	0.24	0.31	0.13	0.16	0.24	0.32	0.00	0.00
Thymus	$\bar{X}$	0.02	0.03	0.00	0.01	0.00	0.00	0.00	0.00
	SD	0.02	0.03	0.00	0.00	0.00	0.00	0.00	0.00

TABLE 12 (cont)

COMPARATIVE BIODISTRIBUTION OF I-125 PEANUT LECTIN AND I-131 $F(AB')_2$

PERCENT INJECTED DOSE INCORPORATED PER ENTIRE ORGAN

		C57/BL Mice Bearing EL4 Tumors			
		24HR(N=6)		48HR(N=4)	
		PNA	FAB	PNA	FAB
Blood	$\bar{X}$	0.50	2.46	0.13	0.57
	SD	0.11	0.65	0.04	0.08
Liver	$\bar{X}$	0.48	0.71	0.26	0.19
	SD	0.05	0.20	0.05	0.02
Spleen	$\bar{X}$	0.10	0.08	0.03	0.02
	SD	0.03	0.04	0.00	0.00
Kidney	$\bar{X}$	0.91	0.74	0.36	0.17
	SD	0.15	0.26	0.11	0.01
Stomach	$\bar{X}$	0.27	0.71	0.12	0.18
	SD	0.11	0.28	0.07	0.07
GIT	$\bar{X}$	0.60	1.42	0.16	0.26
	SD	0.15	0.56	0.04	0.04
Salivary	$\bar{X}$	0.10	0.15	0.02	0.02
	SD	0.07	0.06	0.02	0.01
Muscle	$\bar{X}$	0.02	0.06	0.01	0.01
	SD	0.00	0.03	0.00	0.00
Tumor	$\bar{X}$	0.50	1.82	0.24	0.65
	SD	0.27	0.97	0.29	0.66
Lung	$\bar{X}$	0.08	0.21	0.03	0.04
	SD	0.02	0.04	0.01	0.01
Sex	$\bar{X}$	0.06	0.11	0.02	0.01
	SD	0.04	0.07	0.01	0.01
Thyroid	$\bar{X}$	0.53	0.61	0.15	0.19
	SD	0.56	0.61	0.15	0.19

TABLE 13

COMPARATIVE BIODISTRIBUTION OF I-125 PEANUT LECTIN AND I-131 F(AB')$_2$

PERCENT OF INJECTED DOSE INCORPORATED PER GRAM TISSUE

CAB/CAJ Mice Bearing RI Tumors

		8 HR(N=3)		24HR(N=4)		48HR(N=5)		72HR(N=1)	
		PNA	FAB	PNA	FAB	PNA	FAB	PNA	FAB
Tumor	$\bar{X}$	4.80	3.32	1.30	0.52	0.33	0.16	0.16	0.092
	SD	2.53	0.17	0.18	0.04	0.09	0.03	0.00	0.00
Blood	$\bar{X}$	1.38	6.00	0.20	0.89	0.06	0.26	0.03	0.114
	SD	0.30	0.41	0.02	0.09	0.00	0.02	0.00	0.00
Liver	$\bar{X}$	1.07	2.31	0.35	0.40	0.24	0.18	0.21	0.129
	SD	0.08	0.25	0.03	0.10	0.02	0.02	0.00	0.00
Spleen	$\bar{X}$	9.24	2.20	1.92	0.34	0.88	0.13	0.62	0.083
	SD	1.57	0.12	0.24	0.04	0.03	0.02	0.00	0.00
Kidney	$\bar{X}$	3.00	13.01	2.69	2.00	1.21	0.74	0.82	0.481
	SD	0.50	1.05	0.15	0.11	0.11	0.06	0.00	0.00
Stomach	$\bar{X}$	9.73	15.69	1.23	1.93	0.38	0.45	0.21	0.091
	SD	3.15	3.03	0.28	0.28	0.05	0.05	0.00	0.00
GIT	$\bar{X}$	1.34	1.88	0.15	0.29	0.06	0.09	0.03	0.048
	SD	0.18	0.12	0.03	0.05	0.00	0.01	0.00	0.00
Salivary	$\bar{X}$	6.24	8.24	1.51	0.90	1.30	0.28	1.13	0.074
	SD	2.02	3.24	0.21	0.21	0.08	0.03	0.00	0.00
Lung	$\bar{X}$	3.06	3.79	0.78	0.96	0.25	0.23	0.10	0.083
	SD	0.28	0.24	0.04	0.71	0.03	0.03	0.00	0.00
Sex	$\bar{X}$	3.17	4.21	0.39	0.49	0.18	0.111	94.412	48.641
	SD	1.01	1.03	0.07	0.19	0.02	0.02	0.00	0.00
Thymus	$\bar{X}$	3.10	5.46	0.68	1.52	0.25	0.49	0.00	0.00
	SD	3.16	5.72	0.22	0.64	0.06	0.10	0.00	0.00

TABLE 13 (cont)

COMPARATIVE BIODISTRIBUTION OF I-125 PEANUT LECTIN AND I-131 $F(AB')_2$

PERCENT OF INJECTED DOSE INCORPORATED PER GRAM TISSUE

		C57/BL Mice Bearing EL4 Tumors			
		24HR(N=6)		48HR(N=4)	
		PNA	FAB	PNA	FAB
Tumor	$\bar{X}$	0.34	1.27	0.18	0.50
	SD	0.08	0.34	0.15	0.29
Blood	$\bar{X}$	0.39	1.92	0.12	0.53
	SD	0.06	0.45	0.04	0.09
Liver	$\bar{X}$	0.43	0.62	0.29	0.21
	SD	0.02	0.14	0.09	0.05
Spleen	$\bar{X}$	0.76	0.62	0.26	0.15
	SD	0.06	0.15	0.08	0.03
Kidney	$\bar{X}$	4.28	3.44	2.09	0.99
	SD	0.96	1.07	0.75	0.08
Stomach	$\bar{X}$	1.18	3.17	0.44	0.72
	SD	0.41	1.36	0.18	0.23
GIT	$\bar{X}$	0.25	0.06	0.10	0.16
	SD	0.04	0.18	0.02	0.04
Salivary	$\bar{X}$	0.78	1.23	0.20	0.26
	SD	0.49	0.36	0.14	0.07
Lung	$\bar{X}$	0.59	1.01	0.25	0.28
	SD	0.14	0.30	0.08	0.09
Sex	$\bar{X}$	0.33	0.71	0.23	0.25
	SD	0.04	0.26	0.09	0.15
Thymus					

The specificity index was compared to the CBA/CAJ mice (with the T antigen bearing RI tumors) and C57/Black mice (with the non-T antigen expressing EL4 tumors) using an unpaired t-test. Statistically ($p < 0.01$) higher specificity indices were found in the tumor and spleen of the RI tumor bearing mice, as compated to the EL4 tumor bearing mice at 24 and 48 hours and the salivary glands at 48 hours. None of the other tissue specificity indices were found to be significantly different ($p < 0.01$) between the two animal tumor models.

At 24 hours, the tumor exhibited a specificity index of 11.46 ± 1.14 in CBA/CAJ mice with RI tumors. Therefore, the $F(ab')_2$ fragment, which has a similar molecular weight to PNA but not the specific glycoprotein binding ability of PNA, could only account for about 8.5% of the PNA localization (expressed as a tumor to blood ratio of radioactivity) that occurred in the RI tumor. However, when an animal tumor model was used in which the tumor did not express the T-like specificities, this preferential tumor localization of PNA was no longer evident. Therefore, a specificity index of only 1.34 was observed in the C57 black mice, bearing EL4 tumors, and 75% of the PNA uptake in this tumor is possibly due to nonspcific localization.

These results point to a preferential localization and retention of radiolabelled peanut lectin only within tumor presenting cell surface receptors with which the PNA may specifically interact. A specificity index of 11.5 at 24 hours post-injection shows excellent specificity when compared with other radioimmunodetection studies in animal tumor models. The peanut lectin, therefore, appears to achieve a specific tumor localization at 24 hours, 3 to 4 fold higher than that obtained by some commonly used antibodies such as anti-CEA. The specificity index of ^{125}I-PNA versus ^{131}I-$F(ab')_2$ was actually found to decreased from 11.5 ± 1.1 at 24 hours to 8.4 ± 1.2 at 48 hours. A decrease of this localization index has been observed in many radioantibody investigations as the tumor mass becomes

larger. Larger tumors may contain necrotic tissue, which will no longer retain the specifically-binding protein. In addition, if tumors above a certain critical size are rich in extravascular blood, they may show a much larger contribution of nonspecific macromolecular localization to the overall antibody localization mechanism. The average mass of the RI tumors used in the present studies at 48 hours (1.47 grams), was about 50% higher than those tumors dissected at 24 hours (0.97 grams). Changes in (1) the relative ratios of viable to necrotic tissue, or (2) tumor vasculature, may have resulted in the lower specificity indices observed at 48 hours.

Two other tissues examined in these biodistribution studies revealed significantly different specificity indices when comparing the two different animal tumor models. The high specificity index (19 $\pm$ 1.6) observed in the submaxillary glands of CBA/CAJ mice at 48 hours could either be due to localization of radioiodide or radiolabelled peanut lectin. The in vivo hydrolysis of the radioiodide from the proteins was found to be much more extensive for the radiolabelled peanut lectin than for the $F(ab')_2$ fragment. Analysis of plasma samples at 48 hours revealed that only 44 percent of the peanut lectin radiolabel, ^{125}I, was precipitated by TCA while 75 percent of the ^{131}I radioactivity was found to be protein bound in the plasma.

The last tissue examined, which appeared to exhibit differential mouse tumor model distribution of PNA, was the spleen. The multiple functions of the spleen, and the presence of both lymphoid and reticuloendothelial cells, allow several alternative postulations for the specific PNA localization in the spleens of CBA/CAJ mice. The peanut lectin may be interacting with cells within the normal spleen of CBA/CAJ which have receptors for this lectin. The major lymphoid mass of the spleen (white pulp) has easy access to the bloodstream, as it is concentrated around the arterioles of the spleen, in the form of periarterial sheaths.

Although the possibility of some PNA aggregation may have contributed to some splenic uptake, the selective spleen uptake was not parallelled by a selective accumulation in the liver. There was no significant difference in specificity indices of the livers of CAB/CAJ mice and C57BL mice.

It is interesting to note that the animal tumor model, in which the selective spleen uptake occurred, was the one bearing a tumor with PNA receptors. The presence of the tumor may have played an important role in the spleen localization.

Tissue Distribution Studies with SBA and DBA

We have also studied the relative and absolute tissue uptake of soybean agglutinin and Dolichos Biflorus agglutinin after the intravenous injection of ^{125}I-labelled lectins. The tumor specificity of these two lectins appears to provide a tumor-to-blood ratio of at least 5, seventy-two hours after injection. The relative tissue uptake (Table 14) shows that the target organ showing the maximum uptake is the lung in the case of SBA, while both the kidney and liver exhibited maximal concentrations of the DBA compound. Thus, target of different organs, through the use of different lectins carrying the appropriate label, can be utilized in accordance with this invention.

TABLE 13

RELATIVE TISSUE UPTAKES*
(24 Hours)

	PNA	SBA	DBA
Tumor	1.30	0.14	0.45
Kidney	2.69	0.30	5.53
Spleen	1.92	1.15	1.44
Lung	0.78	1.68	0.31
Liver	0.35	0.11	3.37
Salivary	1.51	0.37	0.92

* % Inj. Dose per Gram of Tissue (N=7)

Clinical Trials with Radiolabelled PNA

Peanut lectin was prepared, in an affinity purified form, on a column which had a Synthetic T. hapten (β-D-Gal (1→3)α-D-Gal NAc) attached. The toxicity, pyrogenicity and sterility of PNA and ^{131}I-PNA were tested in a standard manner (U.S.P. methods, Edmonton Radiopharmacy Centre and British Columbia Pyrogen Testing Laboratory, Canada). PNA was iodinated with Na^{131}I using the iodogen procedure and separated from the free iodine by gel chromatography on Biogel-P6-DG. After obtaining informed consent, eight patients with proven metastatic cancer (3 colon cancer, 2 breast cancer, 2 lung cancer and one adenocarcinoma with an unknown primary source, Table 14), and presumed normal renal function (normal blood urea and serum creatinine, no proteinuria) who had no known history of allergy (specifically to peanuts and iodine), underwent intradermal skin testing with dilutions (0.5 ml of 1, 0.01 and 0.0001 µg/ml) of PNA and commercially available peanut antigen (Abbot Laboratories, U.S.A.). Each patient was given an oral dose of 10 drops of a saturated solution of potassium iodide, to block the thyroid uptake of radioiodine, on five daily occasions, before and during scintiscanning. Prior to imaging studies, measurements of serum total IgE and RAST (antigen specific IgE) to peanut antigen were performed.

Each patient received an intravenous injection of ^{131}I-PNA (dosage range 17-88 µg, range of radioactivity, 37-93 MBq of ^{131}I, specific activity 1.9 MBq/µg (Table 14) and whole-body, external scintigraphy was performed at intervals (3, 6, 9, 24, 48 and 72 hours) following injection of ^{131}I-PNA. Imaging data was acquired using a large field of view gamma camera (GE-400T), interfaced to a computer (A^2 System, Medtronic, Canada). Because abdominal scintiscans, at 3h post injection, indicated rapid sequestration of the radiopharmaceutical in the kidneys, one patient was imaged sequentially with 5 minute frames for 30 minutes following intravenous administration of ^{131}I-PNA.

One subject (patient RW, Table 14) received a dose of ^{131}I-PNA on two occasions, separated by a 2h interval (Table 14) and underwent additional imaging 6 days following the initial injection. Scintigraphic data were stored in the computer in order to process the images.

Whole body retention of ^{131}I was assessed by counting with a Searle Pho-gamma IV scintillation camera in the whole body mode. Venous blood samples were collected at intervals (5, 10, 15, 20, 30, 40 min; 1, 2, 4, 6, 24 and 48 hours) following injection of ^{131}I-PNA in 6 out of 8 patients and urine was collected for 48h in 7 patients, to determine the pharmacokinetics of ^{131}I-PNA. The level of circulating T antigen were determined by solid phase assay in serum samples taken before ^{131}I-PNA administration in 6 out of the 8 patients.

Biodisposition of ^{131}I-PNA

Toxicity to ^{131}I-PNA was not observed and no patients showed evidence of an immediate hypersensitivity reaction to each antigen during skin testing. Measurements of serum total IgE and RAST in the 8 patients were within normal limits. The serum concentration of T antigen was raised in the six patients who were tested (88.3 $\pm$ 10.9 S.D. µg/ml)(Table 14) compared to concentrations in 107 controls without known malignant disease (19.4 $\pm$ 9.4µg/ml). The mean blood concentration of ^{131}I-PNA, as a function of time, following the intravenous injection of varying doses of ^{131}I-PNA in 6 out of 8 patients. There was an initial rapid elimination of ^{131}I-PNA from the blood with a more delayed clearance beyond 6h. The first component of the biphasic blood concentration-time curve showed a mean blood half life (t1/2) of 44.5 $\pm$ 3.0 S.D. min and the second component had a t1/2 of 15.6 $\pm$ 2.0 S.D.h.

Whole body counting indicated that 12 $\pm$ 1.9% S.D. of the total administered dose was retained in the body at 48 hours. At scintigraphy, this activity was found to be present mainly in the kidney, but in three patients there was a relatively low

concentration of ^{131}I in the thyroid and stomach. Urine collections in 6 out of 8 patients indicated that 82.3 ± 5.3% S.D. of the total administered dose of radioactivity was recovered in the first 24h after injection of ^{131}I-PNA with 94 ± 7% S.D. at 48h. Most of the radioactivity (> 75%) in the urine was in a form that could be precipitated by cold 10% trichloroacetic acid, implying that the radioiodine was excreted bound to a polypeptide. Binding studies with asialo GM1-Synsorb (Chembiomed Ltd., Canada) indicated that 40-70% of the urinary radioactivity had retained its binding potential to the synthetic T. hapten. Gel filtration analysis of the urinary excretion products revealed a structurally intact lectin with a molecular weight of 107,000.

The results of the scanning in 8 patients with metastatic cancer are shown in Table 14. Marked renal concentration of the radiopharmaceutical was apparent in all scans. In both patients with breast cancer (RB and SB, Table 14) there was unequivocal visualization of radioactivity at known sites of secondary neoplasm. However, in one patient (RB, Table 14), the activity was noted in a presumed malignant pleural effusion and nonspecific localization of the radiopharmaceutical by diffusion could not be excluded. In contrast, pulmonary metastases were clearly identified on a thoracic scan of a patient with breast cancer (SB, Table 14) and the scintigraphic findings corresponded with the appearances on a chest x-ray. In the two subjects with lung cancer (I.G. and M.Mc., Table 14), the scintigraphic findings suggesting tumor localization were inconclusive.

In one patient (R.W., Table 14) with adenocarcinoma of the cecum, residual activity was identified at multiple sites on abdominal scintiscans at 6 days following initial injection of ^{131}I-PNA. This residual activity was presumed to be due to localization of ^{131}I-PNA in peritoneal metastases, which had been visualized at an earlier laparotomy. On a 24h liver scan,

metastases seemed to be visualized in a patient (A.B., Table 14) with liver deposits secondary to carcinoma of the sigmoid colon.

When lower doses of ^{131}I-PNA were used, tumor visualization was often not apparent (Table 14). It is of importance to note that although certain scans showed promising results for cancer detection in 6 out of 8 patients, other known areas of metastases were not detected (Table 14). Information in the raw scanning data was such that the sites of involvement had to be known prior to processing of the images by filtering and background subtraction techniques. Renal activity and the associated scatter prevented visualization of adjacent abdominal structures, except in one patient (AB, Table 14) in whom the liver was well visualized because the right kidney was located in the right iliac fossa.

To our knowledge, this is the first time that radiolabelled lectins have been administered by intravenous injection to man. Lectins have been used in vivo as carriers for the delivery of chemotherapeutic agents to neoplasms in experimental animals. Injection of concavalin-A conjugated with a chemotherapeutic drug has been found to prolong the survival of tumor bearing mice more effectively than injection of lectin or the cytotoxic drug alone. The results show that the scintigraphic detection of certain forms of malignancy can be effected, especially when doses of the order of 88 µg of PNA are used. In successful radiodetection experiments in animals, approximately 0.7 µg of ^{125}I-PNA (specific activity 500 kBq/µg) were used in mice weighting approximately 25 g. To produce comparable results in man, a dose of approximately 2 mg should have been used. However, in pilot clinical trials, we were limited to smaller doses because of the unknown systemic toxicity of PNA in humans. Animal toxicity data conducted by us indicates that PNA is relatively notoxic in single doses up to 12 grams equivalent in man.

The rapid blood clearance of a tumor seeking reagent, such as PNA, has the theoretical advantage of allowing higher tumor to background ratios for scanning, but if metastases have poor blood perfusion, then rapid excretion and renal concentration of ^{131}I-PNA could lead to suboptimal localization for scintigraphy.

There are many naturally occurring lectins with varying specificity for carbohydrate antigens and it seems likely that other lectins may be useful in the targeting of tissues or perhaps the scintigraphic detection of malignancy. In two patients (AB and SB, Table 14), ^{131}PNA did not image known bony metastases that had been treated with radiotherapy. It is possible that treatment of cancer may alter the expression of tumor antigens, thereby interfering with the tumor seeking potential of a lectin or antibody. Despite the presence of circulating T antigen in our subjects, as measured by solid phase assay, tumor localization of ^{131}I-PNA occurred.

TABLE 14

INTIGRAPHIC AND SEROLOGIC FINDINGS IN 8 PATIENTS WHO RECEIVED ^{131}I-PNA BY INTRAVENOUS INJECTION

tient	(Sex)	Dose of ^{131}I-PNA (µg)	(MBq)	Serum Concentration of T Antigen (µg/ml)	Primary Cancer	Known Metastatic Site	Metastases Assessed by Investigation	Scintigraphy Findings
W.	M	18 17 Total 35	40 29 69	80	Cecum adenoca (not resected)	Liver Surface Omentum Serosa	L.S, L., P.R. C.T, Bi	Peritoneal metastases probably seen on 6 day scan
M.	M	18	38	92	Unknown Adenoca	Liver	L.S., C.T., Bi	Inclear
W.	F	17	37	78	Descending colon adenoca resected	Lung	L., P.R., Bi	Unclear Pulmonary metastases not visualized
B.	F	81	93	88	Sigmoid colon adenoca resected	Liver Lung Omentum Bone	L.S., Bi, C.T., P.R., B.S.	Liver metastases probably seen in 24h scan
B.	F	88	100	--	Breast adenoca resected	Lung (Pleural effusion)	P.R., B.S. Bi	Pulmonary effusion visualized in 24h scan
B.	F	69	64	--	Breast adenoca resected	Lung Bone	P.R., B.S. Bi	Pulmonary metastases visualized in 24h scan

TABLE 14 (cont)

TIGRAPHIC AND SEROLOGIC FINDINGS IN 8 PATIENTS WHO RECEIVED ^{131}I-PNA BY INTRAVENOUS INJECTION

ent	(Sex)	Dose of ^{131}I-PNA (µg)	(MBq)	Serum Concentration of T Antigen (µg/ml)	Primary Cancer	Known Metastatic Site	Metastases Assessed by Investigation	Scintigraphy Findings
	F	71	65	84	Lung (anaplastic epidermoid) not resected	Lung Mediastinum	M., Bi P.R.	Visualization of pleural effusion adjacent to tumor in 24h scan
	M	18	38	108	Lung epidermoid not resected	Lung	P.R., Br., C.T., B.S.	Possible visualization of mediastinal tumor in 24h scan

= liver scan
= plain radiography
= computerized tomography

L = laparotomy
Br = bronchoscopy
Bi = biopsy

B.S. = bone scan
M. = mediastinoscopy

Based upon the above data, it is concluded that:

1) An RI mouse lymphoma cell line, shown to express T-like antigen receptors, was successfully grown in vivo producing an animal tumor model for the investigation of the in vivo localizing capability of T antigen-avid proteins.

2) Anti-T antibodies were radiolabelled in a protected form to provide a radioiodinated protein which bound avidly to a synthetic T immunoadsorbent. A high binding affinity and specificity of radiolabelled anti-T for neuraminidase-treated red blood cells or RI tumor cells was not demonstrable. In vivo biodistribution studies of ^{125}I anti-T antibodies indicated that the extent of anti-T tumor localization could be matched by that of a nonspecific gamma globulin.

3) An affinity purified peanut lectin preparation was radioiodinated and desalted by gel filtration to provide a radiolabelled protein which maintained in excess of 95% of its initial radiochemical purity, when stored at 4°C for two weeks.

4) Radiolabelled peanut lectin exhibited a very specific and avid in vitro binding for T-like cell surface glycoproteins in both RI tumor cells and neuraminidase-treated red blood cells (N'RBC). Radioiodinated preparations of PNA bound, on average, 66% to a one ml suspension of 2% N'RBC and 20% to 5×10^6 RI tumor cells. The binding was readily inhibited in the presence of galactose and could not be demonstrated with control cells, lacking the T antigen.

5) The carbohydrate-binding specificity of ^{125}I-PNA was investigated, using synthetic carbohydrate immunoadsorbents. The lectin was found to have an avidity for the T disaccharide, β-D-Gal(1→3)-GalNAc which was about 50-fold (α-linked), and 40-fold (β-linked) higher than for galactose and at least 10-fold higher than than seen for all other carbohydrates tested.

6) ^{125}I Peanut lectin was found to have good tumor localization and rapid blood clearance, upon i.v. injection in CBA/CAJ mice, bearing RI tumors. Clear images to tumors were

obtained in serial scintigraphic imaging by 24 and 48 hours post-injection. No blood subtraction techniques were necessary for tumor delineation.

7) Biodistribution studies of ^{125}I-PNA in RI tumor-bearing CBA/CAJ mice, revealed tumor:background tissue ratios of radioactivity which increased steadily with time. The tumor:blood and tumor:muscle ratios were 17:1 and 40:1, respectviely, at 48 hours.

8) The in vivo specificity of retention of radioiodinated peanut lectin in RI tumors was verified when ^{125}I-PNA tumor retention was eleven-fold higher than that found with a nonspecific protein $F(ab')_2$ fragment of IgG. Such preferential tumor uptake was not evident in an EL4 mouse tumor model, in which the tumor did not express PNA receptors.

9) A significant spleen uptake of radiolabelled peanut lectin occurred in mice, bearing RI tumors, but was not evident in mice bearing EL4 tumors.

10) Based on the data from in vitro binding investigations and in vivo biodistribution stidies in tumor-bearing mice, radioiodinated peanut lectin is useful in the radioimmunodetection of tumors expressing the T antigen.

11) Other lectins, such as Dolichos Biflorus agglutinin and Soybean agglutinin also localize in tumors and have a particular affinity for certain organs. This is important since we can now target tumors in such organs with the radiolabelled imaging or therapeutic lectin.

12) We have shown that we can successfully, clinically image a number of tumors using radiolabelled PNA.

Claims

1. A composition of matter comprising a lectin selected from a group consisting peanut lectin, lectin extract of orange peel, Maclura pomifera lectin, Dolichos Biflorus agglutinin and Soybean agglutinin conjugated with an agent selected from the group consisting of a therapeutic agent and a radiolabel.

2. The composition of claim 1 wherein said lectin is peanut lectin.

3. The composition of claim 1 wherein said lectin is lectin extract of orange peel.

4. The composition of claim 1 wherein said lectin is Maclura pomifera lectin.

5. The composition of claim 1 wherein said lectin is Dolichos Biflorus agglutinin.

6. The composition of claim 1 wherein said lectin is Soybean agglutinin.

7. The composition of any one of claims 1, 2, 3, 4, 5 or 6 wherein said agent is radioactive iodine.

8. The composition of any one of claims 1, 2, 3, 4, 5 or 6 wherein said agent is technetium-99m.

9. The composition of any one of claims 1, 2, 3, 4, 5 or 6 wherein said agent is conjugated to said lectin through an amino acid residue.

10. The composition of any one of claims 1, 2, 3, 4, 5 or 6 wherein said agent is conjugated to said lectin through polylysine.

11. The composition of any one of claims 1, 2, 3, 4, 5 or 6 wherein said agent is conjugated to said lectin through polyglutamic acid.

12. The composition of any one of claims 1, 2, 3, 4, 5 or 6 wherein said agent is conjugated to said lectin through an amino acid residue and DTPA or EDTA.

13. The process for detecting cancer cells or a malignant tumor in a human which comprises injecting into the human the composition of claim 1 having a radiolabel and scanning the human by scintigraphy.

14. The process for detecting cancer cells or a malignant tumor in a human which comprises injecting into the human peanut lectin having a radiolabel and scanning the human by scintigraphy.

15. The process for detecting cancer cells or a malignant tumor in a human which comprises injecting into the human lectin extract of orange peel having a radiolabel and scanning the human by scintigraphy.

16. The process for detecting cancer cells or a malignant tumor in a human which comprises injecting into the human Maclura pomifera lectin having a radiolabel and scanning the human by scintigraphy.

17. The process for detecting cancer cells or a malignant tumor in a human which comprises injecting into the human Dolichos Biflorus agglutinin having a radiolabel and scanning the human by scintigraphy.

18. The process for detecting cancer cells or a malignant tumor in a human which comprises injecting into the human Soybean agglutinin having a radiolabel and scanning the human by scintigraphy.

19. The process of any one of claims 13, 14, 15, 16, 17 or 18 wherein said lectin is labelled with radioactive iodine.

20. The process of any one of claims 13, 14, 15, 16, 17 or 18 wherein said lectin is labelled with technetium-99m.

21. A dignostic kit suitable for forming a composition useful in identifying a cancer cell or a malignant tumor which comprises a sterile package containing a lectin selected from the group consisting of peanut lectin, lectin extract of orange peel, Maclura pomifera lectin, Dolichos Biflorus agglutinin and Soybean agglutinin and means for mixing the contents of said sterile package with a composition selected from the group consisting of reduced technetium-99m and a physiologically acceptable aqueous solution and radioactive iodine in a physiologically acceptable aqueous solution.

22. The kit of claim 21 wherein the radiolabel is technetium-99m.

23. The kit of claim 21 wherein the radioactive label is radioactive iodine.

INTERNATIONAL SEARCH REPORT

International Application No PCT/US 83/01395

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [3]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int. Cl.[3] A61K 43/00, 49/02, 35/78, 37/46
US. Cl. 424/1.1 128/1.1 260/123.5R

II. FIELDS SEARCHED

Minimum Documentation Searched [4]

Classification System	Classification Symbols
US	424/1.1,177,195 128/1.1,659, 260/123.5R, 112R 436/504,63,64,86,87,91,804,808, 813,827

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [5]

DATABASES: LEXPAT (ALLUTL; 1975-1983) CA Search (1967-1983)

III. DOCUMENTS CONSIDERED TO BE RELEVANT [14]

Category *	Citation of Document, [16] with indication, where appropriate, of the relevant passages [17]	Relevant to Claim No. [18]
X	U.S.A, 4,389,392, Published 21 June 1983, Adachi.	1-23
A	U.S.A, 4,289,747, Published 15 September 1981, CHU.	1-23
A	U.S.A, 4,146,603, Published 27 March 1979, Davidson et al.	1-23
A	U.S.A, 4,334,017, Published 8 June 1982, Plotkin et al.	1-23
X	JP,A, 56-154660, Published 30 November 1981	1-23
X	GB,A, 2,043,890, Published 8 October 1980	1-23
X	JP,A, 57-29950, Published 18 February 1982	1-23
X	JP,A, 57-29951, Published 18 February 1982	1-23
A	N, Biochemical and Biophysical Research Communications, Volume 77, Issued 1977, Pages 581-585, Krejarec et al.	1-23

* Special categories of cited documents: [15]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search [2]	Date of Mailing of this International Search Report [2]
14 December 1983	19 DEC 1983
International Searching Authority [1]	**Signature of Authorized Officer [20]**
ISA/US	Ben K. Padgett

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, [16] with indication, where appropriate, of the relevant passages [17]	Relevant to Claim No [18]
A	N, International Journal of Applied Radiation and Isotopes, Volume 33, Issued 1982, Pages 327-332, Hnatowich et al.	1-23
X,Y	N, Biochemical Journal, Volume 153, Issued 1976, Pages 265-270, Tanner et al.	1-23
X,Y	N, Biochimica et Biophysica Acta, Volume 426, Issued 1976, Pages 688-696, Rittenhouse Simmons et al.	1-23
X	N, Biochimica et Biophysica Acta, Volume 436, Issued 1976, Pages 825-832, Gachelin et al	1-23
X,Y	N, Proc. Natl. Acad. Sci., USA, Volume 73, Issued 1976, Pages 4457-4461, Burridge.	1-23
X,Y	N, Thrombosis Research, Volume 16, Issued 1979, Pages 825-831, McGregor et al.	1-23